THE ISOLATION OF PORCINE ISLETS OF LANGERHANS

by

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Abstract: The Isolation of Porcine Islets of Langerhans

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Chapter One reviews diabetes mellitus and its treatment.

Chapter Two reviews the history and development of islet isolation techniques in both animals and humans.

Chapter Three presents the physiological profile of the pig, its relevance as a human model and results of investigations into porcine pancreatic morphology and histology. Studies into porcine islet isolation are reviewed. Xenotransplantation is reviewed, with a view to the use of pig islets in the treatment of diabetes.

Chapter Four describes the isolation of porcine islets using a manual digestion technique. Results of islet quantification in the digest and the results of islet separation on a bovine serum albumin density gradient are given.

Chapter Five describes the effect of bovine serum albumin osmolality on the separation of porcine islets.

Chapter Six is an experimental comparison of the manual digestion method already described with an automated method. A direct comparison between the two is made using simultaneous digestion by each method.

Chapter Seven describes attempts at the optimization of the automated digestion technique in order to improve the purity of isolate islets, i.e. to improve their behaviour on a density gradient. Modifications to the automated method are described. Each modification and the rationale behind it is described, along with the effects on pancreatic digestion, islet morphology and islet/exocrine separation.

Chapter Eight describes the large-scale isolation of porcine islets after optimized automated digestion as above. The large-scale isolation is performed on a commercial cell separator in which a hyperosmolar bovine serum albumin density gradient, determined by the results in chapter five, is constructed. The effect of the transplantation of such isolated islets into diabetic severe combined immunodeficient (SCID) mice is studied and results given.

Conclusions are discussed and suggestions made for future research.

A full list of references and bibliography is presented.
Statement of Originality

Unless otherwise acknowledged, the work described in this dissertation is my own independent work undertaken in the Department of Surgery at the University of Leicester.

P. Toomey

October 1994
Dedication

This thesis is dedicated to my parents Peter and Rae and to my wife Susan; for their constant love, help and encouragement.

The creatures outside looked from pig to man,
and from man to pig,
and from pig to man again;
but already it was impossible to say which was which.

(George Orwell)
Acknowledgements

I would like to thank the following people and organisations without whose help this work could not have been completed:

The Nuffield Hospitals Foundation and the Department of Surgery, University of Leicester, who funded these studies.

W.H Parker & Sons Ltd for provision and direct access to porcine pancreata.

The staff of the Department of Surgery at the University of Leicester, who always did their utmost to help and guide me during the period of this research. In particular I would like to thank Professor PRF Bell, Mr Nick London and Dr RFL James for guiding the research in a scientific manner. I would also like to thank Harold Contractor, Drew Bennett, Steve Thirdborough, James Wilson, Scott Rose and Bob Chamberlain for their invaluable scientific and technical assistance.
Publication of Work Performed for this Thesis

1. A simple but effective method for the controlled collagenase digestion of the human pancreas.
   London NJM, Toomey P, Lake SP, Wilson J, Bassett PD, Bell PRF, James RFL.
   Transplantation 1990; 49, No 6: 1109-1113.

   Toomey P, London NJM, Contractor H, Bassett PD, Wilson JM, Loftus IM, Bell PRF, James RFL.

3. The effect of osmolality and glucose concentration on the purity of human islet isolates.
   London NJM, Toomey P, Contractor H, Thirdborough ST, James RFL, Bell PRF.

4. Pancreatic islet purification using bovine serum albumin: the importance of density gradient temperature and osmolality.
   Chadwick DR, Robertson GSM, Toomey P, Contractor H, Rose S, James RFL, Bell PRF, London NJM.

5. Human islet purity is greatly improved by separation on hyperosmolar compared to isosmolar density gradients.
   London NJM, Toomey P, Taylor D, James RFL, Bell PRF.
Presentation of Work Performed for this Thesis

1. Pancreatic digestion for islet release: a direct comparison of standard and automated techniques.
   Toomey P, London NJM, Bassett PD, Loftus I, Contractor H, Bell PRF, James RFL.
   British Transplantation Society. Northwick Park Hospital, November 1990.

2. A direct comparison of a standard versus automated pancreatic digestion technique.
   Toomey P, London NJM, Bell PRF, James RFL.

3. The isolation of dendritic cells from the human spleen.
   Thirdborough S, Toomey P, London NJM.

4. Hyperosmolar density gradients and automated pancreatic digestion; improvements towards better islet isolation.
   Toomey P, London NJM, Bell PRF, James RFL.

5. Porcine islet purification: optimization for xenotransplantation.
   Toomey P, Contractor H, Bell PRF, James RFL.

6. A study of the value of intraductal collagenase in raffinose/lactobionate solution for the 24 hr storage of the human pancreas prior to human islet isolation.
   Toomey P, London NJM, Contractor H, Bell PRF, James RFL.

7. Human islet purity is greatly improved by separation on hyperosmolar compared to isosmolar density gradients.
   London NJM, Toomey P, Taylor D, James RFL, Bell PRF.

   Toomey P, London NJM, Bell PRF, James RFL.

   Thirdborough S, Toomey P, James RFL, Bell PRF, London NJM.
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List of Abbreviations

A.D. Anno Domini
B.C. Before Christ
BSA Bovine Serum Albumin
CSII Continuous Subcutaneous Insulin Infusion.
DMSO Dimethylsulphoxide
ELISA Enzyme linked Immunosorbent Assay.
ESRD End-Stage Renal Disease.
FACS Fluorescence activated cell sorting.
FCS Fetal Calf Serum (S-0001a, Seralab, Sussex, UK)
GFR Glomerular Filtration Rate.
HBSS Hanks’ Balanced Salts Solution with 0.35g/l sodium bicarbonate, Northumbria Biologicals, Cramlington, UK.
M812
HEPES 1M buffer solution pH 7.2–7.4. (Northumbria Biologicals Ltd, Cramlington, UK. M932).
HLA Human Leukocyte Antigen.
IDDM Type 1 Insulin-Dependent Diabetes Mellitus.
IEq Islet Equivalent (conversion calculated on the basis of the volume of an islet of mean histological diameter).
i.p. intraperitoneal
IVGTT Intravenous Glucose Tolerance Test.
M Molar
MCBM Muscle Capillary Basement Membrane.
MEM Minimal Essential Medium with Hanks’ salts (Northumbria Biologicals Ltd, Cramlington, UK).
MHC Major Histocompatability Complex.
MI Myocardial Infarction
mmol Millimole.
mOsm Milliosmole/kg of water.
NCS Newborn Calf Serum (AS-202-50, Applied Protein Products, Brierley Hill, UK).
PBS Phosphate buffered saline.
RPMI Roswell Park Memorial Institute – 1640 tissue culture medium, (Northumbria Biologicals Ltd, Cramlington, UK).
<table>
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<tr>
<th>Acronym</th>
<th>Meaning</th>
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<tr>
<td>UW</td>
<td>University of Wisconsin. Viaspan TM (Belzer UW) cold storage solution, (Du Pont Pharmaceuticals, E.I. du pont de Nemours &amp; Company, Wilmington, Delaware 1989)</td>
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<tr>
<td>UK</td>
<td>United Kingdom</td>
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<td>UV</td>
<td>Ultra-violet</td>
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<td>WHO</td>
<td>World Health Organisation</td>
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Introduction & Synopsis

The potential of human islet transplantation as a treatment for patients with Type 1, insulin dependent diabetes has been the subject of much study for nearly twenty years. Recent and on-going clinical trials are showing the feasibility of islet transplantation as a potentially effective treatment, a prospect which is assuming greater importance as the natural history of the disease and the results of currently available treatment become clear.

The world-wide incidence of insulin-dependent diabetes is increasing. Despite the use of insulin and intensive administration regimes, physiological patterns of glucose metabolism are seldom achieved. The long term complications of diabetes remain a major cause of morbidity and mortality amongst the diabetic population, and are thus becoming more prevalent. The operation of whole organ pancreas transplantation provides the best glycaemic control but is beset with complications and still cannot produce completely physiological glycaemic control. The transplantation of human islets of Langerhans is a mode of treatment in which insulin-secreting tissue can be placed in a physiological site, the portal circulation, and exert its effects with minimal risk of complications. Recent reports from the USA, Canada and Italy confirm the production of insulin independence in patients given technically acceptable islet transplants. The long-term effects of course remain to be observed.

With confirmation of the safety and efficacy of human islet transplants is likely to come a large demand for donor islets. Present technology requires the pancreata of three to six cadaveric donors to produce enough islets for an effective transplant. As the world-wide donor organ situation is already critical, it is certain that demand will not be met using human cadaveric donors alone. The answer to this problem must lie in the field of xenotransplantation; to transplant islets from a suitable animal species, under conditions of immunosuppression, immunoprotection or immunomodulation. Porcine insulin bears the closest resemblance to human insulin, and indeed has been the mainstay of conventional diabetic treatment until very recently. Thus, the pig is the most suitable species available to supply tissue for human islet transplantation.

This thesis addresses the problems inherent in the production of pure porcine islets of Langerhans. Porcine islets are morphologically very different to human islets and are considerably more fragile, being easily damaged during the isolation process. This thesis will describe the evolution of an isolation technique for the production of intact, viable, pure porcine islets.
Chapter One reviews diabetes mellitus and its treatment. Special emphasis is placed on the increasing incidence of the disease and the increasing incidence of long-term complications. The pathogenesis of diabetic complications is reviewed as well as the effects of glycaemic control. The efficacy of all currently available treatments are reviewed.

Chapter Two reviews the history and development of islet isolation techniques. All experimental methods and major advances in pancreatic digestion and subsequent islet purification are included, in both animals and humans. The development and current status of the transplantation of purified human islets for the treatment of diabetes is reviewed.

Chapter Three presents the physiological profile of the pig, along with its relevance as a human model. Results of investigations into porcine pancreatic morphology and histology are presented. The history and current status of studies into porcine islet isolation are reviewed. Xenotransplantation is reviewed, with a view to the use of pig islets in the treatment of diabetes.

Chapter Four describes the isolation of porcine islets using a manual digestion technique developed in this department. Pancreatic digest produced by this method is placed on a continuous density gradient of bovine serum albumin for the isolation of purified islets. Results of islet quantification in the digest and the results of islet separation on the gradient are given for ten pancreatic preparations. Details of digestion method, islet counting techniques, density gradient construction and insulin and amylase assays are given.

Chapter Five describes the effect of bovine serum albumin osmolality on the separation of porcine islets. Islets in the pancreatic digest produced by the method described in Chapter Four are separated on continuous density gradients using bovine serum albumin of physiological and various hyperosmolar osmolalities. Results of insulin and amylase assays of gradient fractions, as indices of islet/exocrine separation, are given.

Chapter Six is an experimental comparison of the manual digestion method already described with an automated method using apparatus built in the department. Full details of the new digestion technique are given. A direct comparison between the two is made using the simultaneous digestion by each method, of the two halves of each pancreatic preparation. The results of seven such pancreatic preparations are presented.
Details of islet quantification in the digest produced by each method are given and compared. Islet purification after each digestion method is also compared.

Chapter Seven describes the attempt to optimize the automated digestion technique in order to improve the purity of isolate islets; i.e. to improve their behaviour on a density gradient. Modifications to the automated method are described, to minimize mechanical trauma and islet damage. Modifications to the concentrations of collagenase used are also described. Each modification and the rationale behind it is described, along with the effects on pancreatic digestion, islet morphology and islet / exocrine separation. The results of nine modified pancreatic preparations are presented.

Chapter Eight describes the large-scale isolation of porcine islets after optimized automated digestion as above. The large-scale isolation is performed on a commercial cell separator in which a hyperosmolar bovine serum albumin density gradient, determined by the results in chapter five, is constructed. Full details of the method are given. Results of five consecutive isolations are given, in terms of islet yield and purity. The effect of the transplantation of such isolated islets into diabetic severe combined immunodeficient (SCID) mice is studied and results given.

Conclusions are discussed and suggestions made for future research.

A full list of references and bibliography is presented.
CHAPTER 1


1.2 Diabetic complications. Incidence, mortality and morbidity.

1.3 The pathogenesis of diabetic complications.

1.4 The relationship between the incidence and severity of diabetic complications and glycaemic control.

1.5 The treatment of insulin-dependent diabetes mellitus with exogenous insulin. The effects of insulin regimens and modes of administration on mortality and morbidity.

1.6 The treatment of insulin-dependent diabetes mellitus with the transplantation of insulin-secreting tissue.
Historical Aspects of Diabetes Mellitus

"Diabetes is a wonderful affection, not very frequent among men, being a melting down of the flesh and limbs into urine. The patients never stop making water, but the flow is incessant, as if the opening of aqueducts. Life is short, disgusting and painful; thirst unquenchable; excessive drinking, which, however, is disproportionate to the large quantity of urine for more urine is passed, and one cannot stop them either from making or drinking water. Or if a time they abstain from drinking, their mouth becomes parched and their body dry; the viscera seems as if scorched up; they are affected with nausea, restlessness and a burning thirst; and at no distant time, they expire".

This early description of diabetes mellitus was by Aretaeus of Cappadocia around AD 60. The term "diabetes" was first coined in the 3rd century BC by Appolonius of Memphis and Demetrius of Apamea, to describe a condition of polyuria requiring treatment by dietary restriction. The term originates from the Greek verb "diabaino"- to "go through" and "diabetes" from the thing the fluid runs through i.e. a syphon.

"Overabundant urine may be treated with a mixture consisting of bones, wheat grains, fresh grits, green lead and water." This, the earliest recognition of diabetes was recorded on the 'Ebers Papyrus', discovered in Thebes in 1862. It had lain there since approximately 1500 BC and possibly represents the first known medical text to describe the disease. The mixture above was to be "left to stand moist, strained and taken for four days." The results of this treatment are unrecorded.

The link between 'sugar' and diabetes was first recorded in India by Susruta in 400 BC, who referred to "madhumeha" (honeyed urine) and noted that the urine in cases of "ikshumeha" (sugar urine) was always popular with flies. Treatment at this time relied on elaborate diets, herbal and mineral agents, and exercise.

Galen (AD 29-99), the Greek philosopher and physician, also described a disease likely to be diabetes, referring to it as "diarrhoea of the urine". Many other references, chiefly from Greece and China, are made to it at this time. The Chinese recognised obesity as an important aetiological factor, with diet and exercise the prime forms of treatment. The Persian physician Rhazes, 800 years later, noted the diabetic symptoms of great thirst and exhaustion, and described the complications of loss of sexual function, furunculosis, phthisis and gangrene.
During the 16th century a Swiss physician and alchemist rejoicing in the name of Aureolus Theophrastus Bombastus von Hohenheim (thankfully known as ‘Paracelsus’) pronounced that diabetes was a general disease caused by an alteration of the blood composition. He advised the testing of urine, and indeed evaporated the urine to produce a white powder. This he failed to taste and so mistook it for saltpetre. It is interesting to note that Paracelsus saw only two cases during his lifetime. The lipaemia of diabetes was later described by the Belgian physician Johannes van Helmont.

The modern history of diabetes began when Thomas Willis re-described the presence of glycosuria in certain cases of diabetes, "...the pissing evil", in 1674, and concluded that this must be preceded by the appearance of sweetness in the blood. Willis also distinguished a form of diabetes without sugar in the urine - diabetes insipidus. It was not until 1774 that Matthew Dobson demonstrated that the sweetness in the blood was due to the presence of sugar, which was later shown to be glucose by Chevreul in 1815.

The link between the pancreas and diabetes was first noted in 1682 when Johann Conrad a Brunner observed that removing the pancreas in dogs produced the characteristic signs of the disease. However he did not make a conclusive connection as his experimental animals rapidly recovered due to incomplete removal of the pancreas. The connection was not made until 1889 when von Mering and Minkowski performed a complete surgical removal of the pancreas in a dog. Their attention was drawn to the presence of sugar in the urine by an assistant mentioning that the urine of these dogs attracted flies.

Following this discovery it was assumed that the exocrine secretions of the pancreas in some way prevented diabetes. This was disproved by Minkowski in 1892, showing that dogs did not develop diabetes following pancreatectomy if a portion of pancreas was embedded, with its vascular supply, in the abdominal wall. Removal of the tissue resulted in diabetes, thus proving that the pancreas prevented diabetes by a factor other than the exocrine secretions. These findings led to the suggestion that diabetes was prevented by some form of internal secretion. The same year, 1893, Laguësse suggested that small clusters of cells within the pancreas originally described without knowledge of their function by Paul Langerhans in 1869, might be the source. Laguësse named them the islets of Langerhans. Confirmation that the islets were the source of the internal secretion was provided by Söbelow in 1902. He showed that duct ligation caused atrophy of the exocrine tissue leaving the islets intact and the
animals did not subsequently develop diabetes. Opie (1905) subsequently showed that removal of the duct-ligated gland caused diabetes to ensue.

**Insulin**

At the beginning of this century enormous efforts were made to isolate the endocrine secretion of the pancreas. It was given a name, insulin, by de Mayer in 1909, before it had been isolated. Many extraction methods were tried but produced only preparations that were either ineffective or toxic. Towards the end of the second decade interest was beginning to wane, with most investigators believing that insulin was not extractable. In 1921 Banting and Best began experiments to extract the internal secretions of dog pancreata. It seems that they were largely unaware of much of the previous discouraging work on the subject. They used duct-ligated dog pancreas and used a cold, acidic solution for extraction. By choosing these conditions, for reasons which still remain unclear, they avoided the pitfalls which had caught previous investigators, namely digestion of insulin by exocrine enzymes and denaturation in alkaline solution. Their extract was able to reverse diabetes on injection into diabetic dogs.

Banting and Best adapted their method to extract insulin from calf pancreas and injected their extract into a diabetic patient in 1921 with dramatic results (Banting and Best 1922). Successive effects of available preparations were erratic, but the development of a more reliable large-scale extraction method from the bovine pancreas by Collip led to much better control (Banting et al 1922). Insulin was in general use for the treatment of diabetes throughout Europe and North America by 1925.
1.2 The Complications of Diabetes Mellitus

Type 1 insulin-dependent diabetes mellitus (IDDM) is today defined according to WHO criteria (WHO 1985):
- Fasting blood glucose > 7.8 mmol/l on more than one occasion.
- Hyperglycaemia > 11.1 mmol/l after ingesting a 1.75g/kg (max 75g) oral glucose load.

Incidence, Mortality and Morbidity

The complications of this disease are many. Psychological and psychiatric problems are not uncommon but are beyond the scope of this thesis. The physical complications may be broadly divided into two categories:
1. Acute metabolic disturbances, in effect inadequacies of management.
2. Chronic abnormalities possibly related to the quality of long-term glycaemic control.

Acute metabolic disturbances are mainly episodes of either hypoglycaemia or ketoacidosis. They are the primary cause of death in patients with IDDM below the age of twenty years (NIH 1985). Hypoglycaemia is the commonest complication of insulin therapy and for some patients the most severe problem associated with the diabetic state (Schade 1981). One in three patients experiences hypoglycaemic coma at some time during insulin therapy, about one in ten during the course of an average year and about one in thirty experiences recurrent problems with severe hypoglycaemia. The mortality of ketoacidosis has of course dramatically declined with advances in therapy such as intravenous insulin, volume replacement with saline and potassium supplementation. However, the mean mortality rate is still 9%, with a wide range from 0-20% in major centres and rates often as high as 20-30% in non-specialized units (Felts 1981). Ketoacidosis accounts for up to 16% of diabetic deaths (Tunbridge 1981).

The chronic complications have, of course, only come to light because of an increased life expectancy with the use of intermittent subcutaneous insulin. It was soon noted, however, that the life expectancy of diabetic patients was not matching that of their non-diabetic peers. These patients were dying not only of well recognized pathological events and lesions, but were also susceptible to some previously unknown.

Diabetes is a global health problem. In European populations the age-standardized prevalence for Types 1 and 2 diabetes is 3-10% of the population (King 1993), with Type 1 diabetes accounting for approximately 15% of these cases. Europe has the
highest incidence of Type 1 insulin-dependent diabetes, in Finland, at forty three cases per hundred thousand inhabitants. The lowest incidence is in Israel, six cases per hundred thousand, and there is a general increase in incidence in Europe as one proceeds north (Green 1992). The incidence in the UK is half that of the northern European studies, at seventeen cases per hundred thousand. The incidence of diabetes is increasing, and is now approximately three times the incidence in the 1950's (Burden 1989). This gives support to the theory that Type 1 diabetes mellitus has only really become a prominent disease during the last two hundred years (Bottazo 1993). In 1989, 301,670 patients were receiving insulin in the UK, with 1,690 new diabetic children under 16. Today, approximately 0.3% of children in the UK can be expected to develop the disease before the age of 16.

Each year in the UK, 40 000 patients die with diabetes and approximately 20 000 of these are dying as the result of having diabetes (Fuller 1983). Mortality rates for white male and female patients with IDDM are five and eleven times greater than for non-diabetics and rates for black patients with IDDM are twice those for whites (NIH 1985). Premature death is more marked in women than in men and much more obvious in younger age groups. About 12% of patients with IDDM die within twenty years of its onset (NIH 1985). It is particularly apparent in deaths from myocardial infarction (MI), strokes and peripheral vascular disease (Shenfield, 1979). The two most common causes of death in diabetics under the age of 50 are MI and renal failure (Tunbridge 1981).

### The Chronic Complications of Diabetes Mellitus

<table>
<thead>
<tr>
<th>Complication</th>
<th>Risk Increase</th>
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<tbody>
<tr>
<td>Blindness</td>
<td>x2.5</td>
</tr>
<tr>
<td>Cataract</td>
<td>x5</td>
</tr>
<tr>
<td>Renal Failure</td>
<td>x17</td>
</tr>
<tr>
<td>Ischaemic Heart Disease</td>
<td>x2</td>
</tr>
<tr>
<td>Cerebrovascular Accident</td>
<td>x2</td>
</tr>
<tr>
<td>Peripheral Vascular Disease</td>
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The incidence of clinical athero sclerotic disease is increased two to three times with the presence of diabetes (Kannel 1979). The risk of dying from MI for a diabetic man is almost twice that of a non-diabetic man and almost three times for a diabetic woman. In patients under forty five years the increased risk of dying from MI is five times more likely in men and over eleven times more likely in women (Fuller 1983). The presence
of diabetes confers up to a seven-fold increased risk of dying in previously healthy patients suffering an MI. (Singer 1989), and diabetic men of 45-64 years of age have a greater chance of dying from MI than non-diabetics in the age group 65-74. The risk of dying from a stroke is similar but not so marked. Women are twice as likely to die from strokes, men about one and a half times. The risk of occurrence of stroke in diabetics is two to six times greater than in non-diabetics (NIH 1985).

Renal failure is possibly the most major life threatening complication in IDDM of juvenile onset (Andersen 1983). Patients dying of renal failure before 50 years of age, are forty times more likely to have diabetes (Medical Services Study Group 1981). However, as treatment has improved over the last forty years, the incidence of nephropathy and kidney failure has decreased by about one third (Kofoed-Envoldson 1987, and Krolewski 1985). It is a common complication of diabetes - present in seven to ten per cent of diabetics of all ages (Gatling 1986, NIH 1985) with a peak incidence at sixteen years of disease, altogether affecting 30-40% of Type 1 diabetic patients (Kofoed-Envoldsen, 1987). The one year survival of diabetic patients treated with dialysis and transplantation is about 75% and 82% respectively (NIH 1985).

Retinopathy affects 30% of all diabetics (Houston 1982) with an individual risk of development of early retinal changes at nine years of IDDM (Burger 1986). About 1.2% develop changes every year (Foulds 1983). Diabetes is the most common cause of blindness in people aged 45-64 years, and diabetics in this age group are twenty three times more likely to become blind than their non-diabetic peers (BDA 1988). Over the age of 65, they are twice as likely to go blind (Ghafor 1983).

Neuropathy to some extent is present in 60% of all diabetics and causes symptoms in approximately 30% (BDA 1988). Its presence correlates with increasing age, duration of diabetes and male gender (DCCT 1988). In combination with peripheral vascular disease, peripheral neuropathy often causes foot ulceration, which is fifty times more common than in non-diabetics (Peacock 1985).

Peripheral vascular disease manifest as intermittent claudication is two to three times more common in those with diabetes and when the two are present, this at least doubles the risk of subsequent stroke, coronary disease and heart failure. (Brand 1989). Over the age of 65, those with diabetes are twenty five times more likely to have a leg amputated than non-diabetics (Waugh 1986) and diabetes accounts for 40-45% of all non-traumatic amputations (NIH 1985). It has been shown that gangrene of the feet and legs was over forty times more frequent in diabetic than in non-diabetic patients
(Bell 1952). Of all patients with peripheral vascular disease and gangrene, 60-80% have diabetes (Bell 1957). Today, diabetic men and women have a ten and fourteen-fold higher risk, respectively, for a lower extremity amputation (Siitonen 1993).

1.3 The Pathogenesis of Diabetic Complications

**Diabetic Nephropathy**

Diabetic nephropathy as defined by a urinary protein excretion >500mg/24hrs occurs in approximately 40% of IDDM patients (Andersen 1983), 75-80% of whom will eventually progress to end-stage renal disease (ESRD) and require either dialysis or transplantation for survival (Deckert 1981). In diabetic patients, renal disease follows a predictable course from onset of microproteinuria to nephrotic syndrome and finally to renal failure or death. Duration of diabetes is known to be a risk factor for the development of nephropathy, with a peak incidence after sixteen years of disease (Krolewski 1985). Uraemia usually develops within four to six years of the onset of proteinuria and ESRD follows within a year (Herman 1985).

At the time of diagnosis of diabetes, both kidney size and glomerular filtration rate (GFR) are increased (Mogensen 1976). Glomerular volume and capillary surface area are greater than normal, as a result of increased pressure within the glomerular capillary. This phenomenon is also thought to be important in the later progression of diabetic renal disease (Hostetter 1982). These early changes can be reversed by restoration of glycemic control with insulin (Mogensen 1976).

The glomerulus is also the site of the lesions present in later diabetic nephropathy (Gellman 1959, Hatch 1961). Various stages of glomerulopathy are undergone, suggesting that the course of diabetic renal disease is a continuum of glomerular injury. The hallmarks of diabetic glomerular lesions ('glomerulosclerosis') are thickening of the basement membranes of glomerular capillaries and diffuse mesangial expansion (Osterby 1974, Mauer 1984). Proteinaceous materials, including albumin, IgG, fibrin and platelet-degradation products are deposited in the mesangium accounting for its diffuse expansion and volume increase; this seems to be the most important factor in the later development of renal failure (Mauer 1984). Mesangial thickening, which occurs at the expense of capillary filtration surface, initiates the decline in function of the nephron (Steffes 1989).
A five-stage progression of diabetic nephropathy towards ESRD has been proposed (Mogensen 1983):

1. **Renal Hypertrophy and Hyperfunction**  
   Changes on diagnosis as outlined above.

2. **Renal Lesions Without Clinical Signs**  
   Early basement membrane thickening and mesangial expansion without proteinuria. Develops during first two or three years of diabetes.

3. **Incipient Nephropathy**  
   Microproteinaemia, detectable only by radioimmunoassay. Develops in 40% of patients by seven to fifteen years of diabetes. Probably predictive of development of clinical nephropathy (Chavers 1989).

4. **Clinical Diabetic Nephropathy**  
   Persistent dip-stick detectable proteinuria. Usually in association with hypertension (Mogensen 1983) and retinopathy (Deckert 1978). Mortality risk is significantly increased (Andersen 1983). Develops in 40% of patients by forty years after diagnosis.

5. **End-Stage Renal Disease**  
   Develops in 75% of the above cases during the following ten years (Krolewski 1985). GFR declines at an average of 10ml/min per year and hypertension increases (Osterby 1975; Parving 1983).

The pathogenesis of diabetic nephropathy is by no means clearly understood, but three major theories are currently proposed: metabolic, haemodynamic and genetic mechanisms.

**Metabolic**

Studies have shown the following phenomena with microvascular complications as the consequence of either hyperglycaemia or relative insulin deficiency:

- A decreased incidence of nephropathy and retinopathy with good glycaemic control (Engermann 1977, Hanssen 1986).
- Correlation of basement membrane thickening with known duration of metabolic disturbance (Kilo 1972).
10

- Development of glomerulosclerosis in kidneys from non-diabetic donors transplanted into diabetic patients (Mauer 1976(a)).
- Reversal of nephropathy in donor kidneys from diabetics transplanted into non-diabetic recipients (Abouna 1983).
- Regression of proteinuria in diabetic animals by insulin treatment or islet transplantation (Mauer 1978, Cohen 1987).

Haemodynamic

The haemodynamic theory of diabetic nephropathy pathogenesis is based on the fact that its progression is closely related to the presence of hypertension. Hypertension is uncommon at the time of diagnosis of diabetes mellitus but by the age of 24 years, its prevalence is already higher than in non-diabetics (Christlieb 1981). Diabetic patients with hypertension but without proteinuria are at greater risk for the subsequent development of frank nephropathy (Jensen 1987). It is unclear from the many animal and human studies whether hypertension does or does not initiate the onset of nephropathy. Haemodynamic theory suggests an increase in renal plasma flow and glomerular transcapillary hydraulic pressure in the diabetic kidney. With duration of the diabetes, these haemodynamic alterations lead to direct cellular injury, causing a proliferation of mesangial cells and matrix, resulting in glomerulosclerosis (Hostetter 1982, Zatz 1986). This theory is, however, by no means completely accepted (Bank 1987).

Genetic

The genetic theory of pathogenesis remains controversial and largely unresolved. Studies measuring the muscle capillary basement membrane (MCBM) width of diabetic and non-diabetic twins have not shown reliable statistical differences (Ganda 1983, Barnett 1983), although a difference was found in a study comparing the MCBM width between the non-diabetic parents of insulin-dependent diabetic children (Marks 1981). Familial susceptibility to nephropathy has been shown in patients with Type 1 diabetes mellitus (Seaquist 1989) but consistent associations with HLA markers have not been found. The association of a family history of hypertension with nephropathy in patients with insulin-dependent diabetes may reflect genetic susceptibility (Viberti 1987, Krolewski 1988).

It seems that metabolic, haemodynamic and genetic factors all play a role in the development of diabetic nephropathy. Patients with genetic predispositions towards
Diabetes are more vulnerable to vascular damage in the presence of only mild to moderate hyperglycaemia than those without genetic predispositions. The presence of a family history of hypertension may indicate an underlying genetically determined renal susceptibility to hypertension and diabetic nephropathy in patients with insulin-dependent diabetes.

**Diabetic Neuropathy**

Known risk factors associated with the development of neuropathy are a young age of onset and long duration of diabetes (Knuiman, 1986). The feasibility phase of the Diabetes Control and Complications trial (DCCT Research Group 1988) also suggests risk factors of age (older), and male sex.

The mechanisms by which age and gender might influence the development of diabetic neuropathy are unclear. It has been suggested (Pickett 1982), that the normally slower nerve conduction in men might render them more susceptible to various forms of neuropathy, although this theory remains speculative.

Some major metabolic abnormalities have been implicated in the slowing of nerve conduction as well as in the pathogenesis of other late complications of diabetes mellitus. Hyperglycaemia induces these abnormalities by the slow, non-enzymatic glycosylation of proteins (Brownlee 1984). This changes their properties leading to the accumulation of glycosylated end products, causing increased cross-linkage of collagen, development of brown fluorescent pigments, increased stiffness and decreased thermal stability. This in turn leads to functional and structural alterations in enzymes and membrane walls, leading to abnormalities such as impaired conduction in peripheral nerves (Brown 1984). These phenomena result in a direct association between nerve conduction velocity and hyperglycaemia (Gregersen 1967, Graf 1979, Gregersen 1968, Pietri 1980, Troni 1984).

Hyperglycaemia also induces changes in intracellular metabolites, particularly in the polyol pathway. In the hyperglycaemic state of diabetes, excess glucose is metabolised to fructose by the polyol pathway:

\[
\text{Glucose } \rightarrow \rightarrow \rightarrow \text{Sorbitol } \rightarrow \rightarrow \rightarrow \text{Fructose}
\]

- **NADPH**
- **Aldolase reductase**
- **Sorbitol dehydrogenase**
Thus, in contrast to non-diabetics, sorbitol is produced at a greater rate. Sorbitol does not readily permeate cell membranes and intracellular sorbitol accumulation occurs.

Sorbitol accumulation may lead to alterations of cell function by three mechanisms:

1. **Accumulation of osmotically active sorbitol & fructose.**
   Osmotic damage is particularly implicated in the development of cataracts (Greene 1987). Lens swelling leads to rupture and opacification.

2. **Decreased myoinositol concentrations.**
   Myoinositol is a polyol similar to glucose. The mechanism of its relative deficiency in hyperglycaemia is still unclear, but it is important for maintenance of the Na⁺/K⁺-ATP-ase pump. Deficiency leads to disruption of cell membranes, with, for example, slowing of nerve conduction in peripheral nerves (Zimmerman 1989).

3. **Altered cellular redox potential.**
   Increased polyol pathway activity alters the NADPH: NADP⁺ ratio, and oxidation of sorbitol depletes NAD⁺. These changes lead to reduced protection against oxidative damage at the cellular level (Collier 1992).

Aldolase reductase inhibitors can reverse the above metabolic abnormalities and their place in treatment is about to be tested in a series of prospective trials. Four potent inhibitors have been discovered. Tolrestat (Alredase), Ponalrestat (Statil) and Epalrestat (ONO 2235) which are carboxylic acids, and sorbinil (CP45, 634), a spirohydantoin. These drugs will be tested, together with the effect of myoinositol and gamma-linoleic acid dietary supplementation.

Microangiopathy and resulting nerve hypoxia may also play a part in the aetiology of neuropathy. Sural nerve biopsies in patients with severe progressive diabetic neuropathy, despite good glucose control, have shown small vessel disease (Timperley 1976). Endothelial hyperplasia and capillary blockage are significantly more common in diabetic patients with neuropathy, than in those without and in both are more common than in control subjects (Dyck 1985). A variety of rheological abnormalities have been reported in diabetic neuropathy (Simpson 1988). The severity of both rheological and microvascular factors is correlated with the severity of neuropathy (Dyck 1985, Simpson 1988). The likely importance of hypoxia in the aetiology of neuropathy is also shown by studies in which oxygen supplementation can reverse the abnormalities in diabetic nerves (Low 1988). In these studies the microangiopathy and
endoneurial hypoxia can be seen to be present within 4 months of the onset of diabetes (Tuck 1984). The exact factors in diabetes responsible for the changes leading to hypoxia are as yet unknown.

**Diabetic Retinopathy**

Diabetic retinopathy is usually divided into two stages. In the first 'background' stage microaneurysms and other retinal lesions such as hard exudates occur. This is followed by the 'proliferative' stage in which new vessels and connective tissue grow on the retina, the optic nerve head and into the vitreous.

Diabetes has a great effect on the pericyte population of retinal capillaries, with pericyte loss being much more marked in retinal capillaries than elsewhere in the body (Addison 1970). Pericyte loss is followed by capillary closure. This is often secondary to occlusion with platelet aggregates (Ishibashi 1979). However, retinopathy is capable of developing without conditions of increased platelet aggregation (Kern 1984). The lumen of sites of capillary cell loss is invaded by neuroglia (Eingerman 1989).

The exact pathogenesis is still unknown. However, the metabolic abnormalities listed in the preceding section are also thought to be instrumental in the development in the above lesions of retinopathy. The effects of glycaemic control and aldolase reductase inhibitors are the subject of several on-going trials.
Despite the discovery of insulin in 1922 and the rapid introduction of its use in the treatment of diabetes mellitus, the effects of the disease remain devastating as a result of the long-term microvascular, macrovascular and neurological complications. Because the complications are common to both insulin-dependent and non-insulin-dependent diabetes mellitus, which share elevated blood glucose levels as a primary feature, it has long been suspected that hyperglycaemia plays a primary role in the pathogenesis of those complications.

Epidemiological studies reveal that approx 25% of diabetic patients do not develop complications, irrespective of glycaemic control (Paz-Guevera 1975, Oakley 1974). These and other studies have led to the development of two hypotheses for the development of diabetic complications - the genetic and metabolic hypotheses. The genetic hypothesis is that some patients will develop complications as a result of their genome irrespective of glycaemic control. The metabolic hypothesis suggests that complications are a direct result of imperfect glycaemic control.

**Genetic Hypothesis**

The capillary basement membrane width in the normal offspring of two overtly diabetic patients has been shown to be thickened in over 50% of cases (Siperstein 1968). The incidence of HLA-DR4 antigen has been found to be significantly increased in patients with background and proliferative retinopathy when compared to diabetics without retinopathy (Dornan 1982). However this relationship between retinopathy and HLA-DR4 has not been confirmed by other studies (Christy 1981, Gray RS 1982, Johnston 1982). Studies at Minnesota University have shown that diabetic nephropathy occurs in familial clusters (Seaquist 1989).

**Metabolic Hypothesis**

Studies supporting this hypothesis depend largely on the demonstration of immunofluorescent staining of IgG and albumin lining the renal tubular, glomerular and Bowman's capsule basement membranes. This staining appears in rat kidneys after four to six months of hyperglycaemia and when these 'diabetic' kidneys are transplanted into normal rats, the changes disappear. Conversely the kidneys of normal rats develop the staining when transplanted into diabetic rats (Lee 1974). In humans, kidney tissue obtained from diabetic and non-diabetic patients 2-12 years after renal transplantation...
showed significantly greater frequency and intensity of staining in the diabetic patients
(Mauer 1976(b)).

Clinical studies on the relationship between diabetic control and complications have
long been the focus of controversy (DCCT 1988(b), Skyler 1988). In fact most of the
clinical studies on the relationship between diabetic control and complications have been
confounded by difficulties in experimental design. Most have been retrospective and
many non-randomised. Before the availability of glycosylated haemoglobin as an index
of control there was no acceptable standard, and the end points for defining diabetic
complications lacked precision. The diabetes control and complications trial (DCCT)
should provide the answer. In this trial 1441 people with insulin dependent diabetes
aged 13–39 take part in a proposed 10 year study covering 29 centres in North
America. Half receive conventional insulin treatment whilst the other half are placed on
an intensified management regimen. Close monitoring for the development of diabetic
complications is performed.

Preventing the complications of diabetes has proved to be an elusive goal, and attempts
to reverse clinically manifest retinopathy, nephropathy and neuropathy with improved
glucose control have so far been unsuccessful. Some randomised controlled studies,
conforming to the above standards, in the USA, Canada and Europe, have failed to
show any consistent significant reduction in the incidence of these complications after
intensive insulin therapy for up to four years (Kroc 1984, Holman 1983, Dahl-
Jorgensen 1986, Lauritzen 1983). These results are discouraging, but not completely
surprising as the sample size and duration of follow-up makes it possible that a real
difference is being missed.

Some other studies, conforming to the above standards, have shown more encouraging
results. The development of nephropathy is twice as likely if glycaemic control is poor
(Pirart 1978). But intensive therapy has been shown to result in correction of
glomerular hyperfiltration (Wiseman 1985) and improvement in urinary albumin
excretion (Feldt-Rasmussen 1986) to such an extent that with two years of strict control
(CSII) the progression of early nephropathy was arrested. In more advanced stages of
diabetic kidney disease however, improved glycaemic control has no effect on
progression or severity of the disease (Bending 1986, Viberti 1983, Tamborlane 1982).

With the use of electrophysiological methods to gauge end points, some recent studies
have shown improvements in nerve function with improved glycaemic control (Ehle
1986). Nerve conduction velocity and vibratory sensation threshold have been shown

Results of studies on retinopathy are still mixed. Most studies on the subject have been retrospective. Pirart (1978) followed 4400 diabetic patients between 1947 and 1973, showing that after twenty five years of disease, 80% of patients with poor control developed retinopathy, as compared to 40% of patients with good control. Another retrospective study (Johnsson 1960) showed that patients with good control developed less severe retinopathy than poorly controlled controls, though the overall incidence of retinopathy did not differ. Prospective studies have only been conducted over the past fifteen years, and the duration of follow-up in any of them is seldom more than three years. Possible benefit in terms of delayed deterioration has been shown at two years (Kroc 1988(b)), although other studies can show no difference even at forty one months (Brinchmann-Hansen 1988). There seems to be a phenomenon of transient deterioration of diabetic retinopathy after initiation of improved glycaemic control (Lauritzen 1983, Kroc 1984). Retinopathy then appears to stabilize by twelve months of such control and by two years of treatment significantly fewer microaneurysms and can be seen in these patients than in conventionally treated controls (Dahl-Jorgensen 1986). Most recent prospective studies have failed to show any significant difference in the incidence or rate of progression of retinopathy with good control (Olsen 1987, Holman 1983, Reichard 1990).

Little direct evidence of the effect of glycaemic control on cardiovascular events in diabetic patients is available, due to the lack of prospective studies. The Steno Group reported three patients on conventional treatment suffering five cardiovascular events, with two deaths, whereas one subject given intensive therapy survived a single cardiovascular event (Feldt-Rasmussen 1991). Improvements in lipid metabolism with better glycaemic control are probably the most consistent findings relevant to the risk of cardiovascular disease. Near-normalization of blood glucose has resulted in significant reductions in levels of plasma cholesterol, triglycerides, free fatty acids and plasma apoprotein (Lawson 1985, Tamborlane 1979, Gonen 1985). These changes can occur within three weeks of normoglycaemia and can be maintained for at least three years (Rosenstock 1987).
Recent work shows that good glycaemic control preserves pulmonary function in IDDM, possibly by prevention of capillary basement membrane thickening (Ramirez 1991).

The results of all the above studies suggest that blood glucose control plays a role in the development of diabetic complications. The data supporting the genetic hypothesis is also compelling. It is likely that the microvascular complications of diabetes mellitus develop as a result of an interplay between these factors; i.e. a given patient has a genetic predisposition to develop complications that makes him/her more or less vulnerable to the effects of hyperglycaemia. It appears that hyperglycaemia is essential to the development of complications, but that susceptibility to tissue damage may vary among patients given similar levels of glycaemic exposure (Strowig 1992, Young 1986).

Any beneficial effect on improving blood glucose control is probably limited to halting, or delaying, the progression of early microvascular disease. At most, improving glycaemic control may stop, or delay, the progression of early nephropathy and neuropathy. The progression of early mild retinopathy may be slowed but this is uncertain. Established complications are not affected by changes in glycaemic control. The effect of glycaemic control on the prevention of diabetic complications was unknown due to the lack of good prospective studies. It is hoped that the DCCT trial and the UK Prospective Diabetes Study will provide this information. It certainly seems so because the DCCT trial has been stopped one year short of its intended ten year duration. This was announced at the Las Vegas American Diabetes Association meeting in 1993 (DCCT 1993). The trial has shown an approximate 60% reduction in the risk of developing retinopathy, microalbuminuria (as an indication of impending nephropathy) and neuropathy over this period, if “near normal” glucose control is maintained, with intensification of insulin therapy. This trial certainly seems to indicate a causal association between chronic hyperglycaemia and the microvascular complications of diabetes mellitus.
1.5 The Use of Exogenous Insulin in the Treatment of Insulin Dependent Diabetes Mellitus

Treatment of diabetes with subcutaneous insulin has saved countless lives through the avoidance of the acute ketotic state. Unfortunately, the subcutaneous administration of insulin does not produce metabolic normality in that it fails to restore in diabetic patients the finely regulated blood glucose levels of non-diabetic individuals (Malone 1976).

**Effect of a Single Dose of an Intermediate-Acting Insulin on Plasma Insulin Levels Through the Day**

Most aspects of subcutaneous insulin administration have quite marked limitations. The slow absorption from this site results in glucose concentrations rising excessively at meal times, to be followed by hypoglycaemia. Erratic absorption produces unpredictable glycaemic control, and the lack of feedback prevents the delivery of insulin according to prevailing glucose concentrations. Also the insulin is not delivered to the liver, its most important target organ, directly, as in the physiological situation.

Once-daily insulin injections first became popular in then 1940's and 50's and by the 1960's had become standard practice in many clinics (Skyler 1988). Despite this unphysiological mode of insulin administration it is often still used for convenience to both physician and patient. Twice-daily insulin administration is probably the commonest method used today, usually with a mixture of a short and intermediate-acting insulin: the 'split and mixed' regime (Schade 1983). However, the intermediate-acting insulin does not sustain its effect during the night, with resulting fasting hyperglycaemia. Attempts to correct it often result in nocturnal hypoglycaemia.
Alterations to this regime, with the addition of pre-bedtime intermediate insulin, did not work. This resulted in the adoption of multiple-dosage programmes, with pre-prandial short-acting insulin and intermediate insulin at bedtime (Nathan 1988). This is a fairly physiological regime, which is easy to understand. Strict control may be achieved in many patients with multiple insulin injections, and acceptance of these regimens has been increased by the use of portable ‘pens’ containing an integral needle and cartridge of insulin (Saubrey 1988).

Needleless jet injectors have been developed but a recent policy statement by the American Diabetes Association concluded that “...insufficient information prevents recommendations for their use being made” (American Diabetes Association 1988). Independent trials are needed to identify the most effective jet injector regimens, the frequency of hypoglycaemia, and the possible formation of antibodies to insulin caused by denaturation of the insulin during jet injection.

Open-Loop Devices
The aim is to obtain normoglycaemia, by the administration of insulin via a device, which administers insulin in pre-programmed amounts according to predetermined insulin requirements. No feedback control is present.

In an attempt to produce a smoother pattern of glycaemic control, continuous ambulatory i.v. infusion of insulin was attempted (Slama, 1974). This was later modified into continuous subcutaneous insulin infusion (CSII) by Pickup et al in 1978, using portable mini pumps. The method was approved by the American Medical Association as an alternative to conventional insulin therapy, in 1985 (American Diabetes Association 1985).

Definite improvements in acute metabolic control, compared to conventional insulin therapy, have been reported with CSII (Calabrese 1982, Nathan 1982, Home 1982). Most ‘long-term’ reports of the efficacy of CSII can only give the results of five to seven year experiences, but do give evidence of improved glycaemic control over these periods (Leichter 1985, Mecklenburg 1985, Knight 1988).

Side-effects such as ketoacidosis, hypoglycaemia and skin inflammation at catheter insertion sites have been reported. Recent work shows that CSII, under the auspices of a diabetic centre specializing in the technique, is safe and acceptable. The incidence of side-effects is acceptable at 0.14 episodes of ketoacidosis and 0.1 episodes of hypoglycaemia per patient per year. Glycaemic control is certainly improved, with at
least a 1% decrease in HbA1c level in those receiving CSII (Chantelau 1989). However, effective CSII will always require constant supervision and great patient commitment, and the results in terms of glycaemic control can be achieved by the use of multiple insulin injections (Pickup 1988).

**Closed-Loop Devices**
Closed-loop devices deliver insulin at a rate which is controlled via a feedback mechanism based on plasma glucose level, to provide in effect an *artificial pancreas*. The major problem at present lies in the development of a reliable and accurate implantable glucose sensor. Miles developed a bedside artificial pancreas, the Biostator (Marliss 1977) but it is the size of an artificial respirator and needs connection via two intravenous lines. All attempts at manufacture of an implantable glucose sensor have resulted in failure due to tissue reaction around the device.

The biohybrid artificial pancreas, in which isolated islets are implanted in a device, protected by a semi-permeable polymer membrane and connected to the arterial and venous systems as a shunt (Monaco 1991), may provide useful glucose control. The main advantage of such a device is that ‘immunoisolation’ of the islets is provided, obviating the need for immunosuppression. Up until now however, the results of implantation into pancreatectomized dogs have not been impressive, due to problems of thrombosis, infection and difficulty in loading an adequate amount of islet tissue (Sullivan 1991). A few dogs in these studies however, were rendered normoglycaemic for up to one year.

Alternatively, islets may be immunoisolated within diffusion chambers (Lanza 1991, Lanza 1992), or microcapsules (Lim 1980, Norton 1988), and placed intraperitoneally (Lanza 1991), subcutaneously (Lacy 1991) or in other sites. None of these methods however, produce normoglycaemia in animal models for greater than three to six months (Lanza 1992 (b)).
1.6 The Treatment of Insulin-Dependent Diabetes by the Transplantation of Insulin-Secreting Tissue

A logical alternative way of obtaining good control of glucose metabolism is to replace the cells that secrete insulin, since these will hopefully retain the homeostatic mechanisms for normoglycaemia. These cells may be replaced as:

2. Isolated Islets of Langerhans.

*Pancreas Transplantation*

The first pancreatic transplants were autografts performed in dogs by Minkowski in 1892. These were studies into the physiological nature of the pancreas, based on revascularisation experiments.

The first recorded human pancreas transplant was performed in 1893 by Williams, on a fifteen year old boy, treated with subcutaneous implantation of three pieces of freshly slaughtered sheep’s pancreas. The boy died, after three days, and histological examination of the graft showed only a "fibrous stroma".

Hedon, in 1893, performed a free autograft of part of the pancreas of a dog, and was apparently able to prevent diabetes after the removal of the remaining pancreas.

The first attempt to transplant human pancreas in order to treat diabetes was made by Pybhus and Durh in 1924. Slices of fresh human cadaver pancreas were implanted into the abdominal wall of 2 diabetic patients, without success.

In 1927 the first successful, immediately vascularised pancreatic grafts were made in dogs by Gayet and Guillaumie (Gayet 1927).

Until the development of insulin, most studies on pancreatic grafts were conducted only as part of investigations into normal physiology, and after its discovery, much interest was lost. However, by the 1950s it was realised that conventional subcutaneous insulin treatment was not having any effect on the development of the chronic complications. Interest in pancreatic transplantation was revived.
Brooks and Gifford (1959) attempted to transplant fragmented neonatal pancreas from stillborn fetuses back to their diabetic mothers' quadriceps muscle fascia. No insulin was produced.

Studies such as those by Lichtenstein and Barschak (1957) described allografts in dogs, and identified the major technical problems of thrombosis and pancreatitis. Later studies such as those of DeJode and Howard (1962), developed techniques of pancreatic transplantation in large animals. These led to the first clinical attempts at human pancreatic transplantation - by Kelly and Lillehei in 1966 at the University of Minnesota (Kelly 1967). Two anaemic Type 1 diabetic patients were given cadaver grafts, of a large pancreatic segment and a whole pancreas respectively. The duct of the segmental graft was ligated. For the whole pancreas graft, the digestive juice was drained into a segment of donor duodenum taken along with the pancreas. In both cases, a simultaneous kidney transplant from the same donor was carried out. In 1970 they reported their experience in ten such cases (Lillehei 1970). Nine grafts functioned immediately and the longest survivor lived eleven months. Insulin was not required in the post-operative period. The first graft failed within a few weeks, due to technical problems mainly related to rejection of the associated kidney transplant. This series showed that normal carbohydrate metabolism could be achieved in at least some patients by pancreas transplantation.

All early attempts at pancreatic transplantation were beset with the problems of the drainage of the exocrine portion of the pancreas, i.e pancreatitis, necrosis, abscess and fistula. The American College of Surgeons / NIH Organ Transplant Registry recorded fifty seven transplants, in fifty five diabetic patients between 1966 and 1977. Only two patients were insulin independent for more than one year. Graft survival was no more than 3% at one year (Gerrish 1977).

Most patients at this time were dying of gross sepsis, as a result of excessive immunosuppression and non-healing of anastomoses. Following the lack of success of these early grafts, whole organ transplantation was largely abandoned. Efforts were directed towards the use of suspensions of pancreatic islets as the source of allograft.

Major difficulties were found in:

a) Obtaining sufficient numbers of islets from a single donor.

b) The problem of portal hypertension with intraportal injection of islet preparations.

c) The lack of success with sub renal-capsular injection (Sutherland 1982).
Frustration with early islet transplantation led some surgeons to re-examine means of performing revascularised whole or segmental pancreatic transplants.

The problem of exocrine leakage has always been the major technical difficulty in successful pancreatic transplantation. A variety of techniques have been tried to deal with exocrine drainage. These may be summarised:

1. **Free Drainage Procedures**
   a) Into the Peritoneal Cavity
   b) Into the Urinary Tract (ureter or bladder).
   c) Into the Gastrointestinal Tract:
      i) into a jejunal Roux-en-Y loop
      ii) into the stomach (paratopic graft).

2. **Duct Occlusion Procedures**
   a) Duct Ligation.
   b) Duct Injection - with Neoprene or Latex.

Direct intraperitoneal drainage was performed by Sutherland in 1979. The procedure was thought feasible, because pancreatic proenzymes are not activated in the absence of enterokinase, and the peritoneum is able to absorb pancreatic secretions. The major problems were post-transplant ascites and peritonitis, as the rate of pancreatic secretion in many patients exceeded their peritoneal absorption rate. Only three out of seventeen grafts functioned for more than two years (Sutherland 1982).

In two cases, segmental grafts were placed in the neck with free drainage by direct cutaneous fistulae. Numerous complications ensued and both were removed (Bortagaray 1970).

In 1971, at Montefiore Hospital in New York, Gliedman introduced the concept of anastomosing the duct of a transplanted pancreatic segment to the recipient’s ureter (Gliedman 1973, Gliedman 1978). The results were not spectacularly successful, with only one out of seven grafts functioning in the long-term. Sacrifice of the ipsilateral kidney was usually necessary.

In the United States today the commonest method employs whole organ transplantation, with anastomosis of the adjacent duodenal segment to the urinary bladder (Lillihei 1970, Sutherland 1992). This technique was modified at the
University of Iowa to make the anastomosis easier (Nhiegm 1987). Complications are mainly those related to metabolic acidosis, as a result of obligatory loss of bicarbonate. Urine analysis allows easy, and early, recognition of graft failure by the assay of urinary amylase, glucagon, exfoliative cytology and neopterin excretion.

In Sweden, the usual method used, is to implant the duct of a segmental graft into an intestinal Roux-en-Y loop (Groth 1980). This has become feasible since the introduction of cyclosporin. The lower dose or even absence of steroids in immunosuppressive regimes allows such anastomoses to heal, without the catastrophic septic complications encountered during early attempts at transplantation with enteric drainage. The segmental graft is revascularised by anastomosis of the recipients iliac, to the donors splenic vessels. The duct is anastomosed end-to-side to a jejunal Roux loop, either intra or extraperitoneally. A stent is often brought through the skin for three to four weeks, to protect the anastomosis and to detect rejection by amylase assay. With concomitant pancreatic and kidney transplants in uraemic patients, graft survival is 69% at one year and 60% at three years. In non-uraemic patients, graft survival is only 33% at one year (Groth 1990).

Drainage of the duct into the stomach (paratopic graft) with anastomosis of the recipients splenic vessels to the donors splenic vessels is occasionally used in the USA and UK. Recent results on only fifteen patients report a 60% one year graft survival (Calne 1989).

A major advance came in 1978, when Dubemand and his colleagues in Lyon performed segmental pancreatic grafts in dogs and dealt with the pancreatic duct by occluding it with Neoprene (Dubemand 1978). Although the duct-occluded pancreas was afflicted by pancreatitis, islet function was unimpaired, and provided near-normal blood-sugar control on autotransplantation. Clinically, cadaveric segmental grafts, occluded as above, were anastomosed to iliac vessels. Encouraging results with conventional immunosuppression were followed by a study in Cambridge (Calne 1980) substituting prolamine and then latex for neoprene. The main technical problem was primary vascular thrombosis leading to early graft failure, dealt with by distal arterio-venous splenic fistulae. Peri-islet fibrosis resulting from the duct occlusions, may contribute to islet loss from ischaemia.

The number of methods is a testament to the lack of superiority of any one method. The choice of method of dealing with exocrine secretions, seems to depend on the experience of a particular surgical centre. In the United States, the most active centres...
prefer drainage into the urinary bladder, sometimes with a small cuff of duodenum if the whole pancreas is available for transplant. In Sweden, drainage into an intestinal Roux loop is preferred. In Lyon and Munich, the duct is blocked with a nonreactive plastic. Effective dealing with the exocrine drainage has resulted in a great improvement in both mortality and graft survival rate following pancreatic transplantation.

Results of Exocrine Drainage Technique
(Sollinger 1991, Sutherland 1991)

<table>
<thead>
<tr>
<th>Drainage Type</th>
<th>Graft Survival 1 yr</th>
<th>Technical Failure Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bladder Drainage</td>
<td>65%</td>
<td>14%</td>
</tr>
<tr>
<td>Ductal Injection</td>
<td>56%</td>
<td>25%</td>
</tr>
<tr>
<td>Intestinal Drainage</td>
<td>52%</td>
<td>23%</td>
</tr>
</tbody>
</table>

Of course the introduction of cyclosporin (Calne 1979) was a major factor in improving the results of all organ transplants. Interest in segmental and whole organ transplantation resumed by 1978 and since that time there has been a doubling of the number of grafts every other year. From 1966 to 1984, 501 grafts were performed. Between 1966 and 1988, 1394 transplants were performed (Sutherland 1989).

From 1966 until the most recent Pancreas Transplant Registry Report, May 28 1991 (Sutherland 1992), 3207 transplants have been performed worldwide

Number of Pancreas Transplants
Reported to the Pancreas Transplant Registry since 1977

![Chart showing the number of pancreas transplants reported since 1977, with a peak in 1991.](image)
Pancreas graft survival rates are and patient survival rates are improving continually. In the USA between 1987 & 1990, these rates were 77% and 92% respectively, and outside the USA are 63% and 88%. Graft survival rates are significantly higher in recipients of simultaneous pancreas / kidney transplants, 77%, versus rates of 54% in recipients of pancreas after a kidney, and 52% in those receiving a pancreas alone (Sutherland 1992). In non-uræmic patients it is not logical to accept the side effects of immunosuppression simply to achieve insulin independence. Pancreas transplantation alone in the absence of renal failure is reserved for those few unusual patients who have frequent, severe metabolic complications, or have clinical and emotional problems with exogenous insulin therapy (Sutherland 1992). The results continue to be best in uræmic recipients of a simultaneous kidney with a bladder-drained pancreas. HLA matching confers a small beneficial effect in graft survival (Sutherland 1991).

Immunosuppression centres around cyclosporin and azathioprine, sometimes with the use of anti-T cell agents such as OKT3 or anti-lymphocyte globulin. The effect of FK506 is still to be adequately assessed. The use of University of Wisconsin (UW) solution is now widespread (Ricordi 1992), conferring a much better effect on organ preservation than Euro-Collins solution or hyperosmolar citrate (HOC).

A major objective of pancreas transplantation has always been to prevent, halt or reverse the chronic complications of diabetes. Unfortunately there is no evidence that it does so (American Diabetes Association 1992). It does aim to improve the quality of life with obviation of the need for exogenous insulin injection and rigorous monitoring of blood glucose levels. There are now several hundred patients with functioning grafts, and indeed they exhibit striking reversal of the familiar metabolic abnormalities of insulin deficiency. Exogenous insulin can be withdrawn and fasting glucose levels return to normal within one month of surgery (Sutherland 1990). Glycosylated haemoglobin levels are usually normalized by three to six months, and remain normal, up to ten years so far (Robertson 1991), with survival of the graft. No other method of intervention in the treatment of diabetes can equal this prolonged evidence for normal mean glucose levels. In the Diabetes Control and Complications Trial so far, the mean glycosylated haemoglobin levels in the intensive treatment group, using CSII, remain above normal (DCCT 1987).

Some subtle abnormalities are present however. Although standard oral and intravenous glucose tolerance tests are usually normal, around-the-clock profiles of plasma glucose with full meals are above normal (Sutherland 1984). Plasma insulin and C-peptide, in both plasma and urine are often raised on comparison with non-diabetic controls (Diem 1990). This peripheral hyperinsulinaemia probably reflects an
effect of systemic venous drainage, and a degree of insulin resistance induced by steroids. Recovery from induced hypoglycaemia is subnormal, with respect to both plasma glucose and glucagon levels (Robertson 1989). Levels of plasma lactate and glycerol are higher in pancreas-kidney recipients, than kidney-alone recipients without diabetes (Ostman 1989). Whether these persisting apparently minor deviations from normal physiology will have any ultimate significance remains unresolved. Peripheral hyperinsulinaemia has been shown experimentally to accelerate atheroma (Stout 1981) but no proof yet exists for a parallel effect in the recipients of pancreas transplants. A Swedish study has however shown a trend towards a more atherogenic profile in such patients, with a 50% increase in the LDL/HDL : Cholesterol ratio (Bolinder 1991).

The effect of successful grafting on the late complications of diabetes depends on the complication studied. The evidence strongly suggests that transplantation has a beneficial effect, on both the onset and progression of diabetic nephropathy (Orloff 1975, Landgraf 1989). Patients receiving solitary kidney grafts show a significantly thicker glomerular basement membrane on serial biopsies, compared to those receiving simultaneous pancreas-kidney grafts (Bohman 1985). Serial kidney biopsies, in patients receiving pancreas transplants also show a decrease in the rate of accumulation of mesangial material (Bilous 1989). Studies such as those above are good evidence of improvement, or at least no further advance in diabetic renal complications. The major indication for pancreas transplantation today is in diabetic patients with ESRD who have or who plan to have a kidney transplant (ADA 1992).

The effect of pancreas transplantation on diabetic neuropathy is less dramatic. A study in Stockholm (Solders 1987), using conventional electrophysiological methods, failed to show any significant difference in peripheral nerve function that could not be attributed to an improvement in uremia. No improvement in autonomic nerve function could be shown. Some small but significant improvements in motor nerve conduction velocity have been shown in non-uraemic patients with functioning pancreas grafts (van der Vliet 1988), but most studies show little or no effect on diabetic neuropathy. It has been observed that patients with severe autonomic neuropathy have a higher mortality than those without. A successful pancreas transplant in these patients reduces this risk but any subsequent loss of a functioning transplant confers the greatest mortality of all (Navaro 1990).

Pancreas transplantation appears to have no effect at all on the alleviation or progression of diabetic retinopathy. No studies yet have managed to analyse the 1500 cases found to be necessary as a result of the wide variation in severity of retinopathy, for statistical
significance in the photocoagulation trial (Diabetic Retinopathy Study 1981). A comparison of patients with functioning grafts against those in whom the graft failed within 4 months after transplant (Ramsay 1988), showed no difference in the state of retinopathy after 4 years of follow-up. However the same study showed that those patients with stable retinopathy and a functioning graft showed a marked deterioration in retinopathy if the transplant later failed.

The concept of vascularised pancreas transplantation for the treatment of diabetes does appear unsound, since it is illogical to transplant an organ where 99% of the tissue is unwanted, and capable of producing serious and indeed lethal side-effects. Even if the operation becomes safer it will always remain a major procedure, and therefore justifiable only in patients with established severe diabetic complications. The evidence suggests that it is an unjustifiable treatment for diabetic patients before the development of complications, and the effect of transplantation on established complications is only of proven benefit in diabetic nephropathy, though not on overall renal function.
CHAPTER 2

2.1 Early Experiences in Animal and Human Islet Isolation & Transplantation.

2.2 Islet Isolation and Transplantation in Large Animals.

2.3 The Purification of Animal and Human Islets.

2.4 Islet Storage, Immunomodulation and Immunoprotection.

2.5 The Transplantation of Dispersed Human Pancreas.

2.6 The Isolation and Transplantation of Purified Human Islets.
2.1. Early Experiences in Animal and Human Islet Isolation & Transplantation

The first report of islet isolation was by Bensley (1911), who used microdissection to obtain islets from the guinea-pig pancreas. Although this method was elaborated by both Norberg (1942) and Hellerstrom (1964), the number of islets produced was small (80 islets/mouse), and studies were limited to morphological examination and biochemical analyses to prove islet viability.

A crucial advance in the technique of islet isolation was the use of the enzyme collagenase, to disrupt the architecture of diced pancreas and liberate morphologically intact islets (Moskalewski, 1965). This initial technique was subsequently improved by Lacy and Kostianovsky (1967), who described distension of the rat pancreas with balanced salt solution to facilitate resection, and mincing prior to incubation in collagenase. A yield of 75-100 isolated islets per pancreas was obtained from the digest, predominantly by the handpicking of individual islets with a wire loop. Alternatively, islets were purified by centrifugation, after layering of the digest on a discontinuous, sucrose density gradient. This method resulted in a higher yield of 300 islets per pancreas.

The first report of isolated islet transplantation in the rat was by Younoszai (1970), who transplanted isologous islets into the peritoneal cavity of five diabetic rats and demonstrated a reduction of hyperglycaemia in three rats and normoglycaemia, for four days, in one rat. The effect of isolated islet transplantation in the rat was further documented by Ballinger in 1972, who demonstrated survival of grafted islets, prepared from several isologous donors, transplanted either intraperitoneally or intramuscularly into diabetic recipients. Survival of the recipient rats was extended by this treatment, when compared to controls. In addition, diabetes was ameliorated with maintenance or gain of body weight, reduction of glycosuria and, in some animals, complete post-transplantation normoglycaemia.

These initial experiments demonstrated that islets could be effectively isolated from the rodent pancreas, and utilised for the treatment of diabetes. These early experiments also indicated one of the major problems of islet transplantation, that of the ability to isolate large numbers of viable islets with minimal contaminating tissue. Islet isolation is a multistage procedure, involving pancreatic resection, collagenase digestion and islet purification. Much of the more recent research, aimed at optimising yields, has concentrated on improving these individual stages in the isolation process.
The development of an effective method of islet isolation in the rodent during the late 1960's (Lacy 1967), led to anecdotal reports of human islet isolation using the same technique. Ballinger (1972) isolated some islets from the pancreas of a twelve year old boy via this technique of distension, mincing and collagenase distension. Using this same technique, Sutherland (1974) digested thirty adult cadaveric pancreata and demonstrated the isolation of histologically intact islets. Viability was assessed by the islets ability to synthesize protein, but islets were identified only by their appearance on white light microscopy.

The first attempts at formal human islet isolation and transplantation were made by Najarian et al in 1977. These experiments followed on from the work of Sutherland (1976), showing that the human infant pancreas contained a significantly higher ratio of insulin : amylase than the adult pancreas. Islets were isolated from ten cadaver human pancreata (six infant, four adult) by first dispersing the gland by mechanical chopping, and then incubating with collagenase. Islets were separated from exocrine tissue by centrifugation on a density gradient. The number of islets isolated was never actually reported, but the yield was later stated to be less than 5% (Sutherland et al 1981(b)). The purity of the preparation, as assessed by histology, was not good, even though insulin / amylase ratios suggested purification of 28 to 116-fold in three pancreata.

Four transplants of allogeneic isolated islets were performed in diabetic patients already in receipt of a kidney transplant, and therefore immunosuppressed. The islets were transplanted subcutaneously in three cases, and intramuscularly in one case. No evidence of function was shown by any, but no ill effects were observed. This poor result discouraged further human allotransplantation of purified islets until the results of further large animal work were available.

### 2.2 Islet Isolation and Transplantation in Large Animals

Following the successful reversal of diabetes in the rodent by isolated islet transplantation, attempts were made to develop similar models in large animals, mainly the pig, monkey and dog. In large mammals, including man, the pancreas is a compact, fibrous organ. The techniques appropriate to islet isolation in the rodent, i.e. pancreatic distension, mincing and sometimes islet isolation, were usually ineffective. A further difficulty in the experimental use of large animals is the lack of inbred strains. Thus, for isogeneic experiments single donor / single recipient engraftment must be
used. This necessitates pancreatectomy, with all its attendant technical problems. Multiple donors may be used and allogeneic material transplanted, but the assessment of success is difficult, with failure of diabetes reversal being due either to failure of graft function or to graft rejection.

**Canine Islets**

The pioneering work was performed by Mirkovitch and Campich in 1976. Following pancreatectomy in the dog, they reported that diabetes could be reversed by autotransplantation of microfragments (<2mm) of the digested tail of the pancreas. Before transplantation into the spleen, the tissue was prepared by a combination of mechanical chopping and collagenase digestion. Success was reported in twenty out of twenty five transplants performed, and confirmed by the development of diabetes in seven dogs which then underwent splenectomy. Histological examination of the transplanted tissue showed groups of endocrine cells within the spleen, together with clumps of acinar tissue. The suggested key elements of success were the production of tiny pieces of tissue (all less than 2mm in diameter), the omission of islet purification, and the widespread dissemination of the tissue throughout a well vascularised organ.

This work was confirmed with similar studies by Kolb et al in 1977, Kretschmer et al (1977), Hanson et al (1981), Mehigan et al (1981), Dutoit et al (1982) and Alderson and Fairdon (1984). Kretschmer also demonstrated that the duration of the collagenase digestion was critical, with Mehigan also showing that that not only was the process dependent on the batch of enzyme, but that the outcome was markedly affected by the method and degree of pancreatic disruption. A mechanical mincing technique, with production of a "moderate" particle size resulted in a higher degree of graft success than hand mincing, or mincing to a small particle size. With this technique normoglycaemia is permanent but the IVGTT remains abnormal, as does the circulating insulin level (Kretschmer 1977, Alderson 1984).

Despite these successful reports the technique of transplanting unpurified dispersed pancreas has serious side effects. Intraspelnic injection in dogs is frequently accompanied by significant intraplural embolisation of tissue, producing severe systemic hypotension, bradycardia and cyanosis, with portal hypertension often causing death (Dutoit 1982). These features were also noted in studies where the dispersed pancreas was injected directly into the portal vein (Kolb 1977, Kretschmer 1978). The effect was thought to be due to the exocrine contamination, as well as the quantity of tissue injected.
Matas et al. (1977) used a short period of tissue culture to deplete the enzyme content of minced dog pancreas, followed by autotransplantation to the portal vein of partially pancreatectomized, streptozotocin-induced diabetic dogs. Intraportal injection was limited by the development of portal hypertension in every case. Histological appearances were not presented, but a six-fold increase in the insulin/amylase ratio was claimed. Out of fifteen transplanted dogs, seven became normoglycaemic, as did one out of fourteen controls.

Lorenz et al. (1979) claimed successful transplantation of isolated islet allografts. However, histological appearance, number or purity was not stated. Allografts between partially inbred dogs were given intraportally, without immunosuppression. Diabetes was not satisfactorily induced by partial pancreatectomy and low-dose streptozotocin. Claims of success were based on reversal of diabetes for approximately one week after injection; the subsequent slow rise in serum glucose was interpreted as rejection, but could easily have been the effect of an insulin-containing bolus on borderline diabetes.

Horaguchi and Merrell (1981) eliminated the need for mechanical mincing by developing a method whereby the pancreatic ducts were perfused with collagenase. The enzyme was recirculated after escape through any ruptured distal ducts. This was followed by mechanical dissociation in a shaken water bath. The autotransplanted digest reversed the pancreatectomy-induced diabetes in five out of seven cases. A yield of 57% of the insulin content of the pancreas together with a six-fold purification was estimated, but using an unproven method - staining with Turks solution, usually used in the staining of haematological slides. However, the efficacy of this technique has been confirmed by a number of reports including comparative studies by Griffin (1986) and Hesse (1986). This first description of the intraductal administration of collagenase can now be seen as one of the major landmarks in islet isolation technique. This report is also the first to stress the importance of an efficient collagenase digestion on the efficacy of any subsequent transplant.

Warnock et al. (1983) reported a similar, perfusion-based technique to Horaguchi. Normoglycaemia was obtained in nine out of thirteen pancreatectomized dogs following intrasplenic injection of autografts into the splenic vein. Intrasplenic injection directly into the splenic pulp was ineffectual. The islet yield by insulin content of the pancreas was 25%, but the amylase content was also 25%, thus hardly representing the injection of purified islets.
It is interesting to note that preliminary preparation of the pancreas, by duct ligation, to reduce the amount of exocrine tissue by atrophy, was shown to be detrimental to micrograft transplantation (Mehigan 1980, Yamuchi 1985), unlike similar studies in the rat (Keen 1965).

Noel (1982) used overnight culture followed by Ficoll (a synthetic polymer) density gradient separation to purify canine islets, which were transplanted into the spleen. Hyperglycaemia was reversed in three dogs, but the study was marred again by the use of the unsatisfactory model of diabetes using partial pancreatectomy and streptozotocin.

Serial sieving was attempted as a method to improve the purity of canine islet preparations (Griffin 1986, Hesse 1986), with only limited success. Fragment purity was slightly improved, as shown by the insulin to amylase ratio.

Irradiation (Nason 1986) and cryopreservation (Evans 1987) have also been tried as manoeuvres in islet purification, but with negligible effect.

Long et al (1983) reported the isolation of canine islets in puppies made diabetic by total pancreatectomy, and given autografts of 10% pure fragmented islet autografts into the spleen. Only four dogs were transplanted, three of which remained normoglycaemic in contrast to untransplanted controls. Splenectomy was performed on only one animal, with a partial return of diabetes: this being attributed to embolisation of some islets to the liver.

A method employing ductal collagenase perfusion, gentle trituration and density gradient centrifugation was used by Warnock et al in 1988. Sigma type XI collagenase in Hanks solution was perfused through three cannulae into the main ducts for twenty five minutes until the gland became mucoid. After teasing and trituration, the islets were separated on Ficoll density gradients and collected from the 1.045/1.075 and 1.075/1.085 g/cm³ interfaces. The mean number of islets recovered from each pancreas was 123 000. Purity was quoted at 80-90%, but identification was performed by incident light microscopic examination, without dithizone staining. However, pancreatectomy-induced diabetes was reversed in dogs receiving intrasplenic or intraportal autografts for up to ten months.

Later similar experiments (Warnock 1988), suggested a minimum dose of 5000 islets of mean diameter 122±μm per kg recipient body weight in order to reverse diabetes. Again, islets were identified without the use of dithizone. This quantity could only be
obtained in 30% of isolations. The digestion procedure was modified (Warnock 1989), omitting a pancreatic ductal flush with Hanks before collagenase perfusion. This resulted in a greater number of islets (2,508/g pancreas), with a smaller mean diameter (110μm) but greater purity after Ficoll separation (91%). This time dithizone staining and electron microscopy were used for identification. A number of 7,000 such islets per kg recipient weight was suggested for reversal of diabetes, when allo and autografts of up to 5,000 islets/kg failed to reverse hyperglycaemia.

The latest work on canine islet isolation (Alejandro 1990) uses enzymatic and mechanical disruption, namely intraductal collagenase distension and trituration, followed by semi-automated purification on bovine serum albumin gradients. After work on human islet isolation using albumin gradients (Lake et al 1987 & 1989a) and their production on a large scale with the COBE 2991 cell processor (Lake 1989b), these techniques were used to produce a mean of 328,000 islets (>30 μm diameter) per preparation, with a mean purity of 86.5%. These islets restored fasting normoglycaemia when autografted into pancreatectomized dogs. A direct comparison during this experiment showed relative purities of islets purified on Ficoll and Dextran gradients to be 68% and 63% respectively.

**Primate Islets**

The pancreata of the commonly used laboratory species, such as the dog and rat differ in macroscopic and microscopic structure from that of humans. This has required modifications in technique of islet isolation from those used on the human pancreas. It has been suggested that the only laboratory animal with a pancreas sufficiently similar to that of the human, is the nonhuman primate (Gray 1990). Steps in the development of islet isolation technique in the primate have closely paralleled those in the human.

Attempts were made to perform transplants of unpurified dispersed pancreas in the baboon. Using a similar technique to that used in the dog, Mieny and Smit (1978) reported intraportal autotransplantation in totally pancreatectomized baboons and diabetes was prevented in three out of four animals.

In a larger series, Nash et al (1981) reported the death of all nontransplanted controls, whilst most autograft recipients survived up to 100 days, but were not entirely normoglycaemic. Recipients of allografts became normoglycaemic but rejected rapidly. Scharp et al (1975) described transplantation of isolated islets in Rhesus monkeys, but used an unproven technique for producing diabetes: 70% pancreatectomy followed by
streptozotocin administration. Islets were prepared in a digestion/filtration chamber, separated on Ficoll density gradients, and given as allografts, with immunosuppression, into the portal vein. The histological appearance, purity and number of islets was not reported. After 3 weeks, glycosuria was only reversed in three out of five monkeys but some improvement in the post-transplantation glucose tolerance tests were noted. No long-term results were reported. Results from this type of animal model are difficult to interpret, because of the inability to determine the contribution made to the overall post-transplantation glucose control, by the remaining pancreas.

Jonasson et al (1977) presented autografts of collagenase-isolated, cultured islets, in young monkeys submitted to total pancreatectomy. No details of the islet preparation were given, except viability assessment using trypan blue exclusion. Intraportal injection of this preparation failed to reverse diabetes. Although insulin production was claimed, no data was given in support.

Following the description of a successful method of human islet isolation by Gray (1984), the same technique was applied by the same authors to the Cynomolgus monkey (Gray 1986). After total pancreatectomy, the body and tail underwent intraductal injection of pre-warmed collagenase, followed by incubation and trituration. Islets were purified on Ficoll density gradient and autotransplanted into the spleen or liver. Islets were identified by their opacity on side illumination, without use of dithizone. An average of 1300 islets per gram of pancreas were isolated, but with a purity of only 5-15%. Although the purity was inferior to that obtained by the same method in the human (20-40%), the intrasplenic autografts reversed diabetes in four out of four animals for six weeks, with immediate onset of diabetes after splenectomy. Of five animals receiving intrahepatic autografts, all became normoglycaemic, and although two grafts failed at four and five months, one graft lasted at least nine months.

Using the same technique, Gray et al (1990) performed islet autografts of variable purities to the renal subcapsular site, spleen and portal vein. Again, islets were identified by their 'characteristic' appearance on white light stereomicroscopy. Transplantation of only 5-10% pure islets beneath the kidney capsule failed to reverse diabetes. No functioning islet tissue was found on nephrectomy. Preparations of 'pure' islets beneath the kidney capsule functioned well. These findings provided support for the theory that exocrine contamination is actually detrimental to islet function in this site. However, long-term function of islets in this site was poor. The injection of 5-15% pure islets into the spleen via the short gastric veins produced
fasting normoglycaemia in three out of four animals, although intravenous glucose
tolerance tests (IVGTT) were all abnormal. After six weeks, splenectomy resulted in
the return of diabetes, and histology showed good graft morphology. Intraportal
injection of 5-10% pure islets resulted in immediate portal hypertension in all animals.
Fasting normoglycaemia was present in all animals by two weeks, but the IVGTT was
abnormal in all animals at six weeks. At three to six months three out of six grafts had
failed, but two grafts lasted two and three years respectively. As the groups in this
experiment were studied consecutively instead of concurrently, it is a pity that a direct
comparison of long-term function was not made between intrasplenic and intraportal
injection.

The results of this study were very encouraging for human islet transplantation,
showing that reasonable glucose metabolism can be achieved using islets isolated from
pancreatic tissue similar to humans, and using an isolation technique known to be
successful in the human. It strongly suggested that long-term function was possible
using the transplantation of an adequate number of pure islets with venous drainage
directly into the portal circulation.

Rabbit Islets
Jolly et al (1982) reported allotransplantation of inbred rabbit islets, using a previously
described, non-enzymatic isolation technique (Hinshaw 1981). Some aspects of this
study were, however, unsatisfactory. No histological examination of the islet
preparation was reported. A remarkable yield of 1.8 million islets per pancreas was
claimed without giving details of islet identification or counting method. Insulin
secretion in response to glucose was estimated by an unproved assay. Transplantation
was by intraportal injection into rabbits, made diabetic by streptozotocin, and given
donor-specific blood transfusion and antilymphocyte serum to prevent rejection. The
duration of normoglycaemia following transplantation was not recorded, but inferred
from the survival time! No untreated controls were present and no dose-response
studies quoted for the effect of streptozotocin in the rabbit.

The developments in the techniques of porcine islet isolation will be reviewed in the
next chapter.
2.3 The Purification of Animal and Human Islets

The later stages of any islet isolation procedure involves the separation of the islets from the unwanted exocrine tissue. Although intact islets can be isolated from the pancreatic digest by handpicking with a finely drawn pipette or wire loop, this is impractical for the purposes of clinical transplantation.

Most of the early work on islet purification was performed on rodent islets, using basic cell separation techniques. These rely on the intrinsic properties of the cellular constituents such as cell size or density to achieve separation (Leif 1964, Shortman 1972). Lacy (1967) described unit gravity sedimentation in a vertical tube to assist islet purification. The method was extremely inefficient and handpicking was still required to achieve purity.

Techniques such as elutriation (Scharp 1985), fluorescence-activated sorting (Gray 1989), monoclonal antibodies, lectins (Winoto-Morbach 1989) and electrophoresis (Hymer 1987), have all been extensively tried. None of these techniques yet produce consistently reproducible results applicable to the purification of large numbers of islets.

The variation in buoyant density amongst different cell types has been appreciated for many years (Leif 1964), and has led to the development of a number of methods for physical separation. One of the simplest methods is that of neutral density separation, in which cells are centrifuged in a dense liquid medium and are segregated into two fractions, one denser and one lighter than the suspending medium. Multiple layered discontinuous density gradients are an extension of this methodology, and allow the separation of different cell types over a wide range of densities. The conditions used for density gradient cell separation must maintain cell viability, and for this reason aqueous solutions are employed, toxic materials and extremes of pH are avoided (Shortman 1972). The principle behind the density gradient purification of islets is that tissue in a density gradient will move to the point in the gradient which is of the same buoyant density as that tissue (isopycnic centrifugation). The principle behind isokinetic density gradient centrifugation is that the separation of tissue depends only on the difference in size between particles. The majority of exocrine tissue is single cell and considerably smaller than isolated islets and will therefore move through an isokinetic gradient considerably more slowly than the islets themselves. In almost all centres today, isopycnic centrifugation is the method of choice.
The initial density gradient used to purify rodent islets used sucrose as the medium (Lacy 1967). An improved yield of islets was seen compared to unit gravity sedimentation, but the islets were badly damaged secondary to the extreme molarity (1.8M) of the sucrose. As a result, Ficoll, a synthetic polymer made by the copolymerization of sucrose and epichlorohydrin, was adopted. Its high molecular weight, high density, low viscosity, low osmotic pressure and non-ionised state were thought to render it an ideal solute for a density gradient. Lindall (1969) reported successful islet isolation with avoidance of the harmful effects of sucrose.

As studies with Ficoll continued, variable successes were found to correlate with different batches of Ficoll (Scharp 1973). It was suggested that as yet unidentified toxins were present, affecting the integrity of the cell membrane. Improved islet viability was reported with dialysed Ficoll (Scharp 1973), but not always by other observers (Nash 1976). As these solutions were all still hyperosmolar, other iso-osmotic formulations of Ficoll were developed, including Ficoll-Hypaque (Tze 1976), Metrizamide (Raydt 1977), Ficoll-Conray (Okeda 1979) and Percoll (Brunstedt 1980, Yamamoto 1981). No formal comparisons between these media have been made. Ficoll remains a standard solute in the construction of density gradient media for islet isolation. Hemhke (1986) compared Dextran with Ficoll, finding no difference.

A density gradient of bovine serum albumin (BSA) was introduced as an erythrocyte fractionation medium in 1964 (Leif 1964), and was subsequently used for separating subpopulations of splenocytes (Sunshine 1978) on the basis of their different densities. The first description of its use in islet purification was by Lake et al, (1986) of the Leicester group, who doubled the yield of rat islets with BSA when compared to Ficoll, and also improved the in vitro insulin release and purity of the final preparation. Further studies showed that single donor : single recipient allotransplantation was possible in the rat with use of BSA (Lake et al, 1987). The isolation of human islets with BSA was described (Lake et al, 1989a). After collagenase digestion of human pancreata (Gray et al, 1984) islets were isolated on a large scale using a discontinuous BSA gradient on a commercial cell separator. BSA has a high molecular weight and therefore exerts a minimal osmotic effect; also unlike Ficoll or Dextran, it does not bind water at high concentrations. This means that it is possible to construct a continuous gradient of changing density but fixed osmolality. An average of 2 600 islets per ml of digest were isolated, with an average purity of 63%. A maximum of 90 000 islets were isolated. Viability was proven with in vitro insulin release and transplantation into nude rats (Lake 1989(b) ), and did not differ from the viability of hand-picked purified islets.
Some other laboratories are now beginning to use BSA (Alejandro 1990) and are improving their results.

Whatever technique of centrifugation is used, purified islets will always contain exocrine tissue (varying in quantity from one isolation to another) of the same size and density as islets. A secondary method of purification will therefore be necessary for some isolations in order to produce highly purified islets. A technique for the purification of rat islets from pancreatic digest has been developed using anti-exocrine monoclonals coupled to Dynabeads (James 1989). The principle behind this technique is that the Dynabeads are magnetic and once the monoclonal antibody attached to them has bound to exocrine tissue, the exocrine tissue can be separated from the islets with a magnet. This technique has yet to be tested in human or large animal islets.

Most other centres performing human islet isolation continue to use Ficoll as the density gradient medium (Warnock 1989, 1991, Scharp 1990, 1991). Recently, an interesting further modification to Ficoll has been tested: Euro-Ficoll. Dialyzed Ficoll is dissolved in Euro-Collins solution and used as the density gradient medium. The osmolality of such a solution is approx 450mOsmol/kg. Studies on the isolation of canine and human islets (Olack 1991) showed an improvement in purity to over 90% in both canine and human islet preparations, and improved recovery to over 70%, all results significantly higher than their centres previous best experience. It is hypothesized that the Euro-Collins solution preserves the exocrine tissue, preventing disintegration and preserving integrity, maintaining a wider tissue density difference between islets and exocrine tissue. Whether the improved results are due to an osmotic effect remains to be determined. Improved porcine islet isolation has also been reported using Euro-Collins (Finke 1991), with good viability shown in vitro and in vivo.

2.4 Islet Storage, Immunomodulation and Immunoprotection.

Cryopreservation
The principle behind cryopreservation is the storage of tissue at liquid nitrogen temperatures (-196°C) where metabolic processes and thus ageing are halted. It is theoretically possible to store cryopreserved tissues for millennia. The cryoprotectant used for islet cryopreservation has been dimethylsulphoxide (DMSO). Islets are equilibrated with 2M DMSO and slowly cooled to -40°C prior to plunging into liquid nitrogen. When required, the islets are rapidly thawed (by approximately 200°C/minute) and placed into tissue culture for two days prior to transplantation. The tissue
recovery after thawing is approximately 80%. Although there are no detailed studies at present concerning the viability of human islets after cryopreservation, it has been possible to successfully autotransplant cryopreserved purified canine islets islets, with the reversal of diabetes and the production of a normal metabolic profile (Evans 1990). Cryopreservation offers many advantages to an islet transplantation programme, including the storage of tissue to allow detailed tissue matching, the banking and transport of tissue between centres, and if necessary the pooling of tissue from multiple donors.

Immunomodulation

Immunomodulation refers to techniques to reduce the immunogenicity of isolated islets. Islet immunomodulation protocols are based on the theory that endocrine cells are minimally immunogenic, and it is the antigen-presenting lymphoid dendritic cells within the islets which are responsible for their immunogenicity (Lafferty 1977). If dendritic cells are deleted (either functionally or physically) from islets, then in theory it should be possible to transplant islets with minimal, or perhaps even no recipient immunosuppression. The procedures used to immunomodulate islets have included gamma irradiation (James 1989), UV irradiation (Lau 1983, Hardy 1984), low temperature (24°C) culture (Lacy 1981, Scharp 1987) and the pre-treatment of islets with monoclonal antibodies against class II positive cells (Faustman 1981).

Immunomodulation has allowed long-term allograft survival in mouse and rat islet transplantation, but has not yet been successfully applied to large animals. Although Scharp et al (1990) cultured their human islets at 24°C for 1 week prior to transplantation, their islet transplant recipients all received immunosuppression and it is therefore not possible to determine the immunomodulatory effect of this low temperature culture.
**Immunoprotection**

Microencapsulation of islets, (Lim 1980), is a novel and promising technique to protect islets from the recipient immune system, whilst allowing normal insulin release in response to changes in blood glucose (O'Shea 1984, Darquy 1985). Islets are encapsulated in a liquid core of sodium alginate surrounded by a layer of poly-l-lysine, outside which is another thin layer of alginate. These alginates are highly biocompatible and elicit a minimal host inflammatory response. Poly-l-lysine is a high molecular weight poly-amino acid which, when layered over the inner alginate core forms a semi-permeable membrane. The pore size of this membrane can be altered by adjusting either the molecular weight, or thickness of the poly-l-lysine layer. Most groups working in this field have found excellent in vitro viability (O'Shea 1984, Soon-Shiong 1990) of encapsulated islets, with insulin release characteristics very close to those of unencapsulated islets. The major hurdle at the present time is biocompatibility because although alginates are highly biocompatible, a chronic inflammatory response is nonetheless produced which slowly causes peri-capsular fibrosis and islet death (Soon-Shiong 1991). Normoglycaemia has been produced in vivo with dogs but seldom for longer than three to four months (Soon-Shiong 1991). Although this technique is extremely promising, further research is being conducted to improve the biocompatibility of these capsules. Alternative approaches to immunoprotection of islets lies with the bioartificial pancreas (Monaco 1991), which has been described in the previous chapter, and with the placement of islets in immunologically privileged sites. It has been suggested that the thymus may be such a site in man, which is a practical possibility for islet transplantation (Posselt 1991). This has yet to be tested.

### 2.5 The Transplantation of Dispersed Human Pancreas

The finding that islet purification was not necessary in the dog (Mirkovitch 1977) encouraged several groups to attempt the transplantation of dispersed human cadaver pancreas. Sutherland et al (1980 b) reported eight transplants of dispersed cadaver pancreas into the portal vein of seven recipients, all of whom already had a renal transplant in situ maintained with immunosuppression. Following transplantation a transient drop in insulin requirement was seen but no patient could be withdrawn from insulin. Despite the injection of large volumes of tissue into the portal vein, no permanent ill-effects were recorded, although portal hypertension of up to 60cm H₂O was noted immediately after injection.
Kolb and Largiader (1980) performed dispersed cadaver pancreas transplants in seven patients; into the portal vein of four, and into the spleen of the other three, with a renal transplant from the same donor being performed at the same time. Six patients received no beneficial effect on their diabetes, with one patient, an infant, being withdrawn from insulin 8 months later. It is a moot point whether the graft was really functioning, or the immunosuppression produced reversal of the diabetic state.

As a separate consideration, much interest was shown in the autotransplantation of dispersed human pancreas, usually in patients undergoing total pancreatectomy for chronic pancreatitis. Of course, many patients receiving such autografts had only undergone subtotal pancreatectomy and thus the absence of diabetes could not be taken as a reliable indicator of graft function.

Najarian et al (1980) performed autotransplantation of dispersed pancreas into the portal vein of ten patients, only one of whom underwent total pancreatectomy. An immediate rise in portal pressure up to 30cm H₂O was noted, but no further ill-effects were described. Of the ten patients, three became insulin-independent (all after subtotal pancreatectomy). One of these patients died of unrelated causes and surviving islet tissue was demonstrated in the liver.

Other reports (Valente 1980, Dobroshke 1978, Cameron 1981, Traverso 1981) described intraportal autotransplants of dispersed pancreas, but in no reports could the pancreatectomy be guaranteed as total, and no data could support the suggestions of functioning pancreatic grafts. Severe portal hypertension was a constant result, causing the death of at least four patients out of a total of thirty in the above studies. The severity of the portal hypertension prevented the injection of the complete graft in many cases. Other life-threatening complications of intraportal injection of unpurified tissue included hepatic infarction, bleeding varices, and liver failure (Walsh 1982, Memsic 1984). The placing of unpurified tissue beneath the kidney-capsule also resulted in major complications i.e. sub-phrenic and peri-renal abscesses (Toledo-Pereyra 1986).

No case of autotransplantation following total pancreatectomy was proven to be insulin-independent, and no case of autotransplantation following partial pancreatectomy was shown to have a functioning graft rather than residual pancreas. The technique was shown to be dangerous as well as ineffective. The encouraging experiments in the dog used normal pancreas. Only one study (Mehigan 1980) used the autotransplantation of fibrotic dog pancreas, and only 2 of 27 grafts were successful, with portal hypertension being a major complication.
2.6 The Isolation and Transplantation of Purified Human Islets

No major clinical trials of human islet transplantation were performed during the early 1980's. Progress was temporarily halted while work was conducted on improving the method of islet isolation and purification.

The first description of the successful isolation of islets from the human pancreas came from Gray et al. in 1984. The basic principle behind islet isolation is the dissociation of the pancreas by the enzyme collagenase, into a cell suspension from which islets can then be purified. The specific advance described by Gray was the administration of collagenase via the pancreatic duct. This results in its distribution around the acini of the gland, such that after incubation at 37°C the exocrine tissue becomes separated from the islets. This digestion stage is critical, because if the pancreas is over-digested the islets are broken down into single cells, whilst if the pancreas is under-digested the islets are surrounded by a rim of exocrine tissue which greatly impairs subsequent purification. The pancreas was digested by the intraductal injection (Horaguchi 1981) of pre-warmed (39°C) collagenase, incubated at the same temperature and disrupted by shaking and aspiration, and trituration. The islets were purified by sieving and centrifugation on Ficoll density gradients. An average yield of 1100 islets/g pancreas was achieved, with a purity of 10-40%. Although islets were identified only by dark ground microscopy, good function was shown by supravital staining, in vitro insulin release in response to glucose, and the reversal of diabetes after transplantation beneath the renal capsule in diabetic (immunologically) nude mice. Although the original description by Gray et al. undoubtedly advanced human islet isolation, the collagenase digestion stage was still difficult to control, and produced variable results.

All subsequent methods have employed the intraductal delivery of collagenase in some way. Kneteman (1986) perfused rather than injected human pancreata with collagenase via the duct, in a similar method to that used by Horaguchi (1981) in dogs. The digested tissue was teased apart and purified by filtration. Approximately 85,000 islets per pancreas were obtained with a purity of 20-40%. Kuhn (1985) also perfused the pancreas with collagenase via the duct and used Velcro strips to retain the partially digested tissue, in an analogous process to the method used by Lacy (1982) for the isolation of beef islets. An average yield of approximately 80,000 islets per pancreas was obtained, but no information was given concerning purity or viability.

Scharp (1985) described a method in which the collagenase-distended pancreas was incubated at 37°C and then passed through a tissue macerator, the final final washing of
the islets being performed in an elutriator. The yield increased to 150 000–250 000 islets per pancreas with a purity of 20–25%. Trials of transplantation of this tissue were performed (Scharp 1987), in six diabetic patients with established kidney grafts. Islet preparations were injected directly into the spleen. Insulin-independence was not achieved, but some function was shown in three of the patients by an increase in C-peptide level and decrease in insulin requirement, of 60-90% for two to three months.

Recent advances have greatly facilitated the collagenase digestion stage of human islet isolation. The first was the rediscovery of dithizone (diphenylthiocarbazone), which binds to the zinc in beta cells and turns a bright red colour, thereby allowing the accurate identification of islets (Latif 1988). The second was the description by Ricordi et al. (1988) of an automated method for human islet isolation. The principle behind this automated method is that the pancreas is distended with collagenase and placed into a digestion chamber through which medium recirculates at 39°C. The chamber is then gently shaken, and samples are regularly taken from the chamber effluent and stained with dithizone. As soon as islets appear, the temperature of the circulating medium is reduced to 15°C and the effluent collected at 40°C. The advantages of this technique are that it is minimally traumatic, and freed islets are immediately removed from the injurious action of collagenase. It has also recently been shown that the collagenase digestion of the pancreas is facilitated by the early injection of collagenase into the pancreatic duct (Socci 1993). The pancreata of young (<30 yrs) donors specifically benefit from early intraductal collagenase distension with significantly increased numbers of viable islets being produced both in the digest and post-purification. It is postulated that the early distension supports the immature fibrous architecture of the pancreatic acini, preventing collapse, with subsequent inability to properly fill the acini with collagenase back in the laboratory.

The second stage of islet isolation is the purification of islets from the pancreatic digest. This is achieved by density gradient centrifugation. This technique relies on the fact that islets are less dense than exocrine tissue. Thus when the pancreatic digest is placed on a density gradient, islets migrate to their area of buoyant density and are separated from exocrine tissue. The density gradient media most commonly used are Ficoll, Euro-Ficoll and bovine serum albumin as discussed previously. The most significant advance in the area of density gradient isolation has probably been the description by Lake et al. (1989), of the COBE 2991 cell separator to establish large-scale density gradients. Using this machine it is possible to process large amounts of pancreatic digest in an entirely sterile fashion. The combination of an automated method of digestion, with large-scale density gradient centrifugation on the COBE cell processor
has allowed the isolation of up to 15,000 islets per gram pancreatic tissue. Once isolated, purified islets can be kept free-floating in tissue culture for up to two months.

Recent advances and improvements in human islet isolation have allowed the introduction of clinical trials of islet transplantation. The potential sites for islet transplantation include the renal subcapsular space, the spleen, and intraportal injection into the liver. The renal subcapsular space initially seemed the most attractive site, but since 1988 there have been a number of studies in large animals (Gray 1990, Cattrall 1989), suggesting that the renal subcapsular site is suboptimal for islet transplantation. More importantly there are now reports (Scharp 1990, Tzakis 1990, Warnock 1991) of successful human islet transplants in which purified islets are injected into the liver via the portal vein (intraportal transplantation). All of the recent successful human islet transplants have used the intraportal site. This has the advantage of avoiding the problem of peripheral hyperinsulinemia with its attendant problem of accelerated atherosclerosis (Stout 1981). Access to an intraportal site is relatively straightforward, usually re-opening the left branch of the portal vein, via the umbilical vein in the falciform ligament, under local anaesthetic, in the umbilicus.

The first description of short-term insulin independence after islet transplantation came from Scharp et al (1990). A thirty six year old female patient with a transplanted kidney in-situ was the recipient. Maintenance immunosuppression was 500mg cyclosporin per day. She received a total of almost 800,000 islet equivalents (islets with an equivalent diameter of 150μm), with a purity of 95%, from two donors, isolated over three days. After seven days culture at 24°C the islets were injected intraportally via the umbilical vein. Antilymphoblast globulin, azathioprine and steroids were added to her immunosuppression. This transplant failed after twenty days, probably because of rejection. Shortly afterwards came the report of the results of a series of nine intraportal human islet transplants (Scharp 1991). One patient achieved insulin-independence for only two weeks, but another, who received approx 13,000 islets per kg body weight, both fresh and cryopreserved, from multiple donors, was still insulin independent at time of reporting, five months after transplant.

In Edmonton, Warnock et al (1991(a)) transplanted two patients, each with approximately 250,000 fresh islets, (3,700 islets/kg), both from single donors. A third patient also received 370,000 cryopreserved islets, from four donors, receiving a total of 10,000 islets/kg. All patients received simultaneous kidney / islet grafts from the same donor. Isolation technique entailed collagenase ductal perfusion, trituration and Ficoll density gradient centrifugation, with purities of 45-75%. Insulin independence
was not achieved in the patients receiving the fresh islets alone. The third patient achieved insulin independence, at least for one month, at time of reporting. Another patient who also received cryopreserved islets from multiple donors, did not become insulin independent. These reports stress the importance of transplanting an adequate amount of islet tissue, \textit{approximately 10,000 islets/kg body weight}. Until improvements in isolation technique yield sufficient tissue from a single donor to treat an individual recipient, islet banking by cryopreservation seems able to facilitate collection of enough tissue (Kneteman 1989). To date, the insulin independent patient remains so at one year (Warnock 1992), with a normal IVGTT, and represents the first case of normoglycemia and insulin independence in a Type 1 diabetic patient treated with islet transplantation.

In Pittsburgh, Ricordi et al (Tzakis 1990) applied the use of the automated technique to transplant islets in nine patients undergoing massive upper abdominal exenteration ('cluster transplants'). These patients were not classically type 1 insulin-dependent but became diabetic after removal of the pancreas as well as the liver, spleen, stomach, duodenum, proximal jejunum and terminal ileum during major cancer surgery. They received islet transplants from the pancreas of the liver donor, or in one case, a third party. Islets were separated by automated digestion (Ricordi 1989), and Ficoll density gradient centrifugation on a large scale, on the COBE 2991 cell separator (Lake 1989). Six patients survived the surgery and after transplantation of 105,000–562,000 islet equivalents, three became insulin-independent. Incidental liver biopsy was performed in one patient, during laparotomy for intra-abdominal abscess. This showed a well preserved viable islet which stained for insulin (Ricordi 1992a). All surviving patients however showed an abnormal C-Peptide response, indicative of transplantation of a sub-optimal amount of islet tissue. It is possible that the insulin-independence effect of this sub-optimal transplantation was due to the large weight loss attendant to the surgery, or even to the parenteral nutrition with solutions containing insulin.

To date, twenty one patients have received intrahepatic islet allografts in Pittsburgh, ten following 'cluster' transplantation for malignancy, four following liver transplantation for cirrhosis and diabetes, and seven received kidney / islet transplants for end-stage diabetic nephropathy (Ricordi et al 1992b). All patients were immunosuppressed with FK 506, and the latter eleven also with steroids. Six patients in the first group became insulin independent, two died of recurrence and one became insulin dependent at ten months. The three insulin independent patients are well at thirteen to sixteen months post-operatively. None of the other patients became insulin independent, despite these transplants containing higher mean numbers of islet equivalents with a
greater degree of purity. It is currently thought that a simultaneous liver graft has a protective effect and that steroid treatment has a detrimental effect on intraportal islet allografts.

Since then two patients in St. Louis, and one patient in Miami, have also achieved 'long-term' insulin independence, up to ten months so far (personal communication). The patients in these centres were diabetic patients with established renal transplants. All of these patients were receiving immunosuppression for their renal transplants and the only changes to their immunosuppression protocol was a reduction in their Cyclosporin dose (to minimise Cyclosporin islet toxicity) with the addition of two weeks anti-lymphocyte globulin. All of these successful transplants have utilised more than one pancreas donor with the most recent St. Louis patient receiving cryopreserved islets. In Milan, one patient achieved insulin-independence for four months, six months after transplantation of 600 000 fresh islet equivalents from a single pancreas. Insulin had to be recommenced after an episode of cardiac failure (Soxci 1992). Three other patients in Milan received approximately 400 000 fresh islet equivalents from one or two pancreata and did not achieve insulin-independence.

Future Prospects
Recent improvements in the isolation of human islets have resulted in greatly increased islet yields (up to 2 million islets per pancreas) and purity. The purity of human islet isolates does however vary (40 - 95% pure) and there is a need for secondary purification procedures. Possible techniques include the use of anti-exocrine monoclonals (Fujioka 1990) or lectins bound to magnetic beads (Winoto-Morbach 1989). An alternative approach is the use of fluorescence-activated cell sorting (Gray 1989). At present however there is not a suitable fluorochrome and the available technology cannot cope with the large volume of digest from a human pancreas. The successful human islet transplants to date have all been intraportal. Large animal studies suggest that intrasplenic transplantation may be preferable (Warnock 1989) and there is a need to investigate this site in the human.

It is unlikely that there will ever be sufficient human pancreata to provide islets for even half of the newly diagnosed cases of insulin dependent diabetes, and there has therefore been much interest concerning the isolation of islets from other species. The pig, in particular, is attractive because of the similarities between porcine and human insulin. Techniques have been developed for the isolation of large numbers of porcine islets
Diabetes is an autoimmune disease (Eisenbarth 1986) and there is concern that islet transplants may be destroyed by a recurrence of the autoimmune disease process. One possible solution to this problem has been suggested by Sutherland (personal communication), who on the basis of a review of the World Pancreas Transplant Registry noted that graft survival was best for HLA Class II (DR) matched and HLA Class I (ABC) mismatched transplants. This is thought to be because classic allograft rejection is Class II restricted, whilst islet autoimmunity is Class I restricted. This data suggests that long-term islet survival may be achieved by the mismatching of Class I and matching of Class II loci. The logistics of this would, of course, be greatly helped by effective cryopreservation. The ultimate aim of human islet transplantation is the treatment of newly diagnosed diabetic children, in order to prevent the long-term complications of the disease. For this approach to be ethically justifiable it will be necessary to develop effective immunomodulation protocols for human islets, such that diabetic recipients require minimal or perhaps even no immunosuppression.

In conclusion, whilst there have undoubtedly been major advances in many areas of human islet isolation, culminating in the recent successful transplants, there remains the need for further improvements so that islet transplantation can regularly be achieved on a 1:1 donor to recipient ratio. Moreover, for the technique to be applied to diabetic children efficient immunomodulation protocols need to be developed. If the incidence of insulin dependent diabetes continues to rise (Burden 1989), then xenotransplantation will be required to provide sufficient islet tissue. If advances in islet isolation technology continue at their present rate, there is every reason to believe that these goals can be achieved.
CHAPTER 3

3.1 Xenotransplantation. Historical landmarks and current status

3.2 The Pig. Basic physiology and similarities to the human. Use as an animal model. Porcine insulin and pancreatic anatomy. The microscopic structure of the porcine pancreas.

3.3 Pig islet isolation. Review of techniques and results.
Interest in xenotransplantation has varied over the past three decades. During the 1960's, as allogeneic transplantation became more successful revealing the shortage of human organs, a number of xenografts from primates to humans were performed. Failure to achieve successful long-term transplantation during this period and the new source of allogeneic organs achieved by the acceptance of brain death, dampened enthusiasm for further clinical trials. The 1970's were relatively quiet years in the history of xenotransplantation. More recently, the further success of clinical allogeneic transplantation has again caused a relative shortage of human organs (UKTS 1989, Lancet 1990). More than one third of those now awaiting liver transplants die for want of a donor organ (Caplan 1992). Well over half of all children born with fatal congenital deformities of the heart or liver die without a transplant due to the shortage of organs. The percentage of those who die would actually be much higher if all potential candidates were on waiting lists. Many Americans are not referred because they cannot afford transplantation, and neither are all those from economically underdeveloped countries. Demand for the limited number of organs is increasing due to the increase in the number of centres capable of offering transplantation. Organs are still lost through reluctance of families to give permission for donation and because of medical reluctance to ask. It can easily be seen why there is renewed interest in the possibility of using organs from other species for transplantation into humans.

Solid organ xenotransplantation in humans has been performed only with primate organs. Kidneys, livers and hearts have been tried. No one-year graft survival has yet been achieved although a nine month graft survival of a chimpanzee kidney has been reported (Reemtsma 1969). Early experiences such as this reported immunological studies stressing the possible role of humoral mechanisms of xenograft rejection. This was suggested by pathological findings, and by the correlation of the appearance of cytotoxic antibody with clinical rejection (Porter 1965, Dewitt 1965).

However, nonhuman organs have been used to support human life. Perfusion of the livers of pigs (Ham 1968) and baboons (Bosman 1968) with human blood has been used, with a success rate of 20% survival (Eiseman 1967) in the treatment of hepatic coma. The most striking conclusion from clinical xenotransplantation is that no long-term success has yet been achieved although successful human allogeneic transplantation is now routinely successful. Two important observations have been made:
1. Nonhuman organs have functioned in human beings to support life.
2. The survival of a number of grafts in patients for some months when immunosuppression was less successful than it is today, suggests that long-term xenograft survival, while difficult to accomplish, is achievable.

The source of the best xenograft tissue is still in question. Nonhuman primates have been used with some success as organ donors for humans; the survival of such grafts appears to be sustained in some cases by administration of immunosuppressive regimens aimed at cell-mediated immunity (Auchincloss 1988, Reemtsa 1989). Enthusiasm over the use of nonhuman primates as donors has waned because of the risk of transmitting lethal viruses to the recipient, the cost, ethical concerns and the substantial problems associated with the genome of primates. Chimpanzees and other nonhuman primates are already in short supply, breed slowly in nature and poorly in captivity, and are often widely infected with pathogens dangerous to humans (Douglas 1970, Woodruff 1970). If xenogeneic organs are ever to represent a plentiful new source for human patients, a species more distantly related evolutionarily, but as physiologically similar, must be considered. The pig has certain advantages as a donor; it has large litters, breeds rapidly and is of reasonable size for human transplantation. The physiological similarities will be described later.

Rejection
Rejection is the greatest problem to be overcome in xenotransplantation. Early studies showed that the more distant the relationship between donor and recipient, the more likely was immediate graft rejection. It was soon recognized that many forms of experimental xenogeneic transplantation were models of immediate antibody-mediated graft rejection (Rosenberg 1971). Most solid xenografts appear to be vulnerable to rejection of this type, via natural preformed xenophile antibody, that adheres to endothelial glycoprotein. Islet transplants are susceptible to immune serum (Perloff 1981) but islets take 10 days to vascularise and thus they are relatively immune to this phenomenon. It seems likely that complement activation also plays a large part in this immediate hyper-acute rejection (Platt 1992) and if the immediate xenophile antibody response is minimal, the islets may 'accommodate', i.e. become vascularised by recipient endothelium, especially if the xenophile antibodies can be removed by plasmapheresis whilst vascularisation is occurring. It is also possible that the species-specific complement activation of hyper-acute rejection may be prevented if 'transgenic' pigs, bearing human complement activation factors are used for xenotransplantation (White 1992).
Xenografts are also susceptible to cell-mediated rejection in a similar manner to that of allograft rejection (Perper 1971). Nude mice often accept xenografts indefinitely, but even using nude animals, it is interesting to note that xenograft survival is significantly longer with closely related donor/recipient species. Cerelli (1970) showed that antithymocyte globulin was less effective in prolonging the life of hamster skin than rat skin onto mice. The vascular endothelial cell appears to be a major target in both the humoral and cell-mediated response during the xenograft rejection (Haisch 1991). Despite many studies it is still unknown whether the greater difficulty in prolonging xenografts is the result of quantitatively stronger cell-mediated rejection, or of qualitatively different, perhaps unrecognized, mechanisms of xenograft rejection. It is perhaps, the central issue in the study of xenotransplantation. In vitro studies of cell-mediated immunity are needed, both to determine the quantitative strength and qualitative aspects of xenogeneic as compared to allogeneic responses. In particular, the relative precursor frequencies of xeno versus allo-reactive immune cells of different functional types remain to be determined. The possible need for xenogeneic antigen processing and presentation on self MHC antigens in order to allow effective recognition, should be analyzed. These studies could determine whether the difficulty in obtaining long-term survival is a reflection of stronger allogeneic-type immune reactions, or whether quite different immunological mechanisms are involved. Results should suggest specific immunosuppressive strategies. Studies on in vivo hyperacute rejection are needed to determine the best method of clearing pre-existing natural antibody.

**Current Status of Porcine Xenotransplantation**

(Niekrasz 1992)

- It is necessary to overcome humoral (vascular) hyperacute rejection by removal of preformed anti-pig antibodies from human plasma (Kirk 1993) prior to transplantation. Plasma exchange or extracorporeal immunoabsorption, as well as antibody neutralization, are under investigation.

- Prevention of the further production of antibodies might be achieved by immunosuppression with currently available drugs or newer agents such as 15-deoxyspergualin, possibly in combination with antithymocyte globulin or pre-transplant lymphocyte irradiation.

- Acute cellular rejection must be overcome using a combination of cyclosporin, azathioprine, steroids and possibly FK-506.

- Blood group incompatibility does not seem to be a significant factor, according to available information with regard to pig blood groups.
A major concern in porcine xenotransplantation is the transmission of pathogens between discordant species. Bacterial disease of pigs include tuberculosis and erysipelas; viral diseases include influenza, and fungal diseases include coccidiomycosis. Microbiological screening of Landrace pigs kept on a standard farm (Bjöersdorff 1992) revealed the presence of the following organisms:

Serology: Toxoplasma gondii
Encephalitozoon cuniculi
Leptospira interrogans
Aspergillus fumigatus (exposure only, no colonisation)
All viral serology was negative.

Blood Cultures: Skin contaminants only.
Staphylococcus epidermidis
Bacilli
Enterococci
Corynebacterium diphtheriae

Figs kept in a slaughterhouse (Marchetti 1992) also harboured:
Moraxella
Acinetobacter
Pseudomonas vesicularis
Pseudomonas fluorescens
Pseudomonas putrida
Candida albicans
Candida parapsilosis
Torulopsis candida

All the above are capable of being isolated from the transport fluid of the resected pancreas. It was shown in this last study that all the above contaminants could be removed from the subsequently isolated islets by washing the pancreas with 20% betadine in the slaughterhouse, rinsing with HBSS and adding penicillin, streptomycin, gentamicin and amphotericin to the culture medium. The islets remained viable as shown by in vitro testing and in vivo reversal of diabetes in diabetic mice. It is of course possible to raise pigs in a germ-free environment if facilities exist.

Common neoplasms of pigs include skin melanoma, lymphoma, cardiac rhabdomyosarcoma. Lymphoma accounts for 46% of all tumours in pigs but the overall incidence of neoplasms in pigs is only 0.004%, in pigs living only to the age of slaughter (four to six months).
Human islet xenotransplantation is likely to use the pig as the source of islets, because of the already proven successful use of porcine insulin in the treatment of diabetes. In the absence of a specific regime for xenograft immunosuppression, immunomodulation and protection methods are likely to be necessary. Encapsulation of islets in a semi-permeable alginate–poly-l-lysine–alginate membrane (Sun 1987) is a technique that has received a lot of attention. However, problems of fibrosis around the capsule preventing effective diffusion, have yet to be overcome. Use of the bioartificial hybrid pancreas (Maki 1991, Monaco 1991) is another alternative, again using protection across a semi-permeable membrane. With the thymus, (Posselt 1981) and testis known to be immunologically privileged sites (Whitmore 1977), it might even be possible to transplant to recipients at these sites as has been successfully performed in the rat (Selawry 1989)

3.2 The Pig.
The pig belongs to the order Artiodactyla, which includes the camel, cow, giraffe and hippopotamus. The family is Suidae and the parent genus and species for the present domestic pig are Sus scrofa, the wild pig of Europe and Sus vitratus, the banded pig of Malaysia. The term hog derives from the Hebrew, meaning to encompass or surround. The origins of the Latin 'suinus' (swine) and middle English 'pigge' are still obscure.

The fortunes of the pig have waxed and waned considerably throughout history. It was initially held in very high esteem, being regarded as sacred on Crete during ancient times (Barloy 1978). As time went on, the status of the pig diminished, possibly because of its universal dietary habits. There is still no evidence to support the view that the pig’s diet is any more disgusting than that of any animal and plenty suggesting that the pig is far less prone to overeating. Much has been written about cleanliness, or lack of it, in this species. Under normal conditions most observers agree that they are quite fastidious preferring a warm, clean dry bed to that which is normally provided for them.

Despite the bad press pigs have received, they continue to perform valuable service to man. As well as providing food, pigs have performed quite bizarre services. One pig was trained to guard a marijuana patch and was reported to have bitten two investigating law officers before being subdued. Pigs have been used in animal herding (Mellen 1952) and respond to skilled ‘hog calling’ themselves. In Texas, a swimming pig named Priscilla saved the life of a drowning child, becoming the first pig
to receive the Stillman award for heroism from the American Humane Association (American Humane Assoc 1985).

The Pig as Animal Model of Human Physiology

It is perhaps in the field of biomedical research that the pig performs its most valuable work outside the provision of food. Historically, swine were the first animals whose use in medical research was recorded in print, Vesalius's Fabrica in 1543. A woodcut showed a boar receiving a transection of the recurrent laryngeal nerve prior to a thoracotomy, to demonstrate a technique of intratracheal resuscitation during pneumothorax.

The main function of the pig in research is as an animal "model", that closely mimics human structure, function and/or behaviour. Held (1983) provided a broader definition: "An animal model is a living organism in which normative biology or behaviour can be studied, or in which a spontaneous or induced pathological process can be investigated, and in which the phenomenon in one or more respects resembles the same phenomenon in humans or other species of animals". Animal models should:

1. Accurately mimic the desired function or disease.
2. Be available to multiple investigators.
3. Be capable of being handled easily.
4. Survive long enough to be functional.
5. Fit available animal housing facilities.
6. Be of sufficient size to provide multiple samples.
7. Be polytocous, providing multiple offspring.

Claude Bernard is reported to have said: "For each kind of investigation, we should be careful to point out the proper choice of animal. This is so important that the solution of a physiological or pathological problem often depends solely on the appropriate choice of animal for the experiment, so as to make the result clear and searching." Although swine are quadrupedal, and not used as extensively as laboratory rodents, they are the most appropriate model for many human functions. They tend to be diurnal, sedentary, and meal eaters with a tendency to obesity and cardiovascular disease. With training and diet restriction, they may become passable athletes - a description appropriate for the human population.
**Physiological Profile of the Pig**

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Value Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Rectal Temperature</td>
<td>39°C (38.6-39.5)</td>
</tr>
<tr>
<td>Mean Respiratory Rate</td>
<td>13 /min (8-18)</td>
</tr>
<tr>
<td>Mean Heart Rate</td>
<td>75/min (65-85)</td>
</tr>
<tr>
<td>Mean Systolic Blood Pressure</td>
<td>170mm Hg</td>
</tr>
<tr>
<td>Mean Diastolic Blood Pressure</td>
<td>108mmHg</td>
</tr>
<tr>
<td>Daily Water Requirement</td>
<td>4.5-6.5 litres.</td>
</tr>
<tr>
<td>Daily Urine Excretion</td>
<td>2.5-4.5 litres.</td>
</tr>
<tr>
<td>Daily Food Requirement</td>
<td>1.5-3.0 kg.</td>
</tr>
<tr>
<td>Hb</td>
<td>12.7g/dl (10-16)</td>
</tr>
<tr>
<td>WBC</td>
<td>14.9 x10³/mm³ (11-22)</td>
</tr>
<tr>
<td>Platelets</td>
<td>404 x10⁹/mm³ (300-700)</td>
</tr>
<tr>
<td>PCV</td>
<td>41.2% (32-50)</td>
</tr>
<tr>
<td>Na+</td>
<td>146mmol/l (135-142)</td>
</tr>
<tr>
<td>K+</td>
<td>6.0mmol/l (4.9-7.1)</td>
</tr>
<tr>
<td>Cl-</td>
<td>103mmol/l (94-106)</td>
</tr>
<tr>
<td>Glucose</td>
<td>4.8mmol/l (3.3-7.5)</td>
</tr>
<tr>
<td>Urea</td>
<td>10.7mmol/l (6-17)</td>
</tr>
<tr>
<td>Earliest Mating Age</td>
<td>9-11 months.</td>
</tr>
<tr>
<td>Cycle Length</td>
<td>21 days (Polyoestrous)</td>
</tr>
<tr>
<td>Sexual Receptivity</td>
<td>2-3 days.</td>
</tr>
<tr>
<td>Mean Gestation</td>
<td>114 days (112-116).</td>
</tr>
<tr>
<td>Litter Size</td>
<td>6-16</td>
</tr>
<tr>
<td>Reproductive Span</td>
<td>3-4 years.</td>
</tr>
</tbody>
</table>

The use of the pig as an experimental animal is facilitated greatly by the fact that it is easy to keep, breeds quickly and easily in captivity and has large litters. Pavlov had an unfortunate experience in his use of pigs as experimental animals; the shrieking of a single pig was so disruptive that he banned it from the lab and declared that all swine were hysterical! Despite this, the use of pigs in medical research has greatly increased over the past twenty years as studies show the anatomical and physiological similarities.

Cardiovascular anatomical and physiological similarities have enabled pigs to become valuable models in cardiac surgery. The distribution of the coronary arteries, the blood supply to the conduction system and the ability to form a collateral circulation following
myocardial ischaemia, is identical to those characteristics in man (Peng 1983, Pluth 1983). The porcine dermis and epidermis are more similar to those of man than those of any other animal (Ordman 1966) making the pig the model of choice in most plastic surgery studies. The pig genitourinary tract is more similar anatomically and physiologically to man than other non-primate models (Russell 1981). Probably because the pig is a true omnivore, it has been described as an almost exact model of the gastrointestinal physiology of man (Argenzio 1980). There are some important anatomical differences, most notably the spiral arrangement of the porcine ascending and transverse colon but it still provides a good model for experimental upper gastrointestinal surgical procedures (Van der Sluis 1982). Swine have been described as the best physiological model for comparison to human infants (Book 1974), with a degree of maturity at birth similar to human infants as well as similar haematological values, growth pattern and dental eruption dates.

In the field of organ transplantation, porcine models have been used extensively in studies on heart, liver, kidney, pancreas and intestinal transplantation (White 1979). The similarity in cardiac anatomies has already been described, but kidney and liver anatomy, physiology and response to immunological manipulation and cold ischaemia make these porcine organs also applicable to transplantation studies (Wall 1982, Kirkman 1979). Of particular interest in the field of islet transplantation, is the fact that pigs have already been used as models for whole organ pancreatic transplantation (Agnes 1980, Gabel 1983). Porcine models were used to show that duct occluded segmental transplants are less satisfactory than open duct techniques. The porcine pancreas is a more suitable organ than that of the other animal used in these studies, the dog. The porcine gland is a retroperitoneal organ and the pancreatic head wraps around the portal vein, whereas in the dog the pancreas is mobile on a long mesentery and the pancreatic head is not related to the portal vein. Also the consistency of the porcine pancreatic parenchyma is more similar to that of the human (Traverso 1987, Turegano-Fuentes 1990).

The main reason for using the porcine model in islet transplantation is of course the great similarity between human and porcine insulin (Figures 3.1 and 3.2). The α and β polypeptide chains are arranged in a two-Zn hexamer crystal in both species but at the β chain C-terminal, residue β30 is threonine in human insulin and alanine in pig. Bovine insulin has two additional amino acid differences in the a chain. Comparison of the crystal structure between pig and human insulin show the structures to be virtually identical, with a difference of only 0.3Å at β30. This is insignificant in terms of the
hormones biological activity, and any difference in terms of immunogenicity stems from the small effect of hexamer packing in the crystal lattice (Baker 1988).

Porcine insulin has been used in the treatment of diabetes for over 50 years; it is effective and most of the reactions to its use - anaphylaxis, lipohypertrophy, urticaria, lipoatrophy and local abscesses, come from subcutaneous administration and are also seen with human insulins. These effects are unlikely to be seen with the internal secretion of insulin from implanted islets.
THE PORCINE PANCREAS

The pancreas extends across the dorsal wall of the abdominal cavity behind the stomach. It is triradiate or triangular as shown:

The pancreas develops from the embryonic duodenum by a dorsal and ventral primordium. The central part involutes during embryonic life, only the dorsal pancreatic primordium with the accessory duct remains, in contrast to most animals in which remnants of both, and both ducts remain. The accessory duct enters the duodenum at the minor duodenal papilla, distal to the major papilla. The duodenal process is attached to the first curve of the duodenum (Flexura portalis) and the splenic lobe is related to the left extremity of the stomach, the dorsal end of the spleen and the anterior pole of the left kidney. The middle lobe is virtually median, and posterior. It is related to the portal vein and mesentery, with a junctional lobe connecting to the duodenal process. The duodenal, middle and junctional lobes form a ring - the annulus pancreatis, surrounding the portal vein. In fully grown animals the mass of the whole gland is 100-150g.

The exocrine and ductal components of the porcine pancreatic histology are almost identical to those of man. The structure of the islets of Langerhans however, differs significantly. Whereas human islets are discrete spherical structures, well demarcated from surrounding exocrine tissue, pig islets are far more irregular. They are irregularly shaped, with peripheral islet cells being directly apposed to acinar cells. Loose vascular channels, not seen in the human, are present, breaking up the cellular components of the islet.
Figures 3.3 to 3.8 demonstrate the microscopic structure of the abattoir pigs used in the experimental studies of this thesis:

3.3 The Isolation and Transplantation of Pig Islets.

If islet isolation becomes clinically applicable, meeting the requirement for islet tissue on the basis of human organ donation will be impossible. With numbers of possible cadaveric donors at 1700 per year in the UK, no more than 900 actual donations could be expected. At present the pancreata of two to five donors are needed to isolate enough islet tissue for a transplant to have a chance of success. Swine could represent an optimal source of islet tissue, because of the similarity between human and porcine insulin, and because of the availability of pig pancreata.

Apart from the problem of xenografting across a major species barrier, a large problem lies in the obtaining of large quantities of intact, viable porcine islets; they are remarkably fragile and easily dissociate into single cells during isolation procedures (Ricordi 1986). Porcine islets are very irregularly shaped, with loose vascular channels within the islet, making it easy for the islets to fragment during pancreatic digestion.

The earliest work on the isolation of porcine islets was performed by Wise (1974). A mechanical method of pancreatic disruption was described. The resulting mixture of endocrine and exocrine tissue showed evidence of insulin secretion in vitro. In no way could this report claim the successful isolation of "islets". No islet numbers were quoted and islet "identification" was assumed rather than performed.

Using a modification of the collagenase technique of rat islet isolation, Sutherland et al (1974) reported islet transplants in pigs after total pancreatectomy, both as autografts and allografts in the intramuscular and intraperitoneal sites. Islets were prepared by collagenase digestion of the resected pancreas and isolated on Ficoll density gradients. The histological appearance of the preparation was not described, and no comment made regarding the purity of the preparation or the numbers of islets transplanted. Prolongation of life was demonstrated (thirteen days versus six days, in non-transplanted controls) but the transplants failed to reverse diabetes.

Wise (1982, 1983) described autotransplantation of islets into the spleen of pigs made diabetic by partial pancreatectomy and streptozotocin treatment. The islets were prepared by an unusual non-enzymic method based on physical separation. No histological examination of the islet preparation was reported, and the number and
purity of the islets was not stated. Islet grafts were either fresh or cryopreserved, and viability assessed by insulin secretion in response to glucose. The results suggested very poor viability. Following transplantation of autogenous cryopreserved islets into the spleen, diabetes was reversed in nine out of fifteen animals, and recurred on splenectomy. Islet tissue could not be found on histological examination of the removed splenic tissue.

The first successful report of pig islet isolation was by Ricordi in 1986, using a modification of the intraductal collagenase perfusion technique, used by Horaguchi (1981) in dogs. The pancreata of slaughter-house pigs were obtained with a warm ischaemia of ten to fifteen minutes. Injection of collagenase was performed to produce distension and the pancreas incubated at 37°C until biopsies showed the beginning of islet release. Digestion was stopped by plunging into cold Hanks balanced salts solution (HBSS). The pancreas was mechanically disrupted by a motor-driven meat grinder and the tissue then filtered to 0.5mm fragments. Islets were purified by centrifugation on Ficoll density gradients similar to those already described for rat islet isolation (Lindall 1969). Islets were identified by their brown colour on green-light microscopy and an average yield of 1215 islets per gram of pancreas was obtained in the initial digest. No details were given regarding any size threshold for inclusion in the count. After purification, qualitative purity figures of 88-99% were given but no figures of ultimate islet yield were given. Some of the post-purification islet numbers were greater than the pre-purification numbers, indicating fragmentation. Yield expressed as insulin recovery from the pancreas was approx 33%. Islets were cultured for six days and showed insulin release in vitro. They also reversed diabetes in mice for three days.

This method was also used in a later study (Ricordi 1987), to produce pig islets for renal subcapsular transplantation in nude mice. No islet numbers were quoted but 10μl of the previous 90-95% pure preparation were implanted beneath the renal capsule of 10 diabetic mice. Only two mice became normoglycaemic, with subsequent nephrectomy showing good islet morphology. Neither mouse lived to exhibit return of diabetes after nephrectomy. Again, this method using the tissue macerator, produced much islet fragmentation.

More recently, Marchetti et al (1988) isolated an average of 400 islets per gram pig pancreas, using a method based on collagenase digestion and filtration of the digested tissue. No purification was used and islets were identified without the use of dithizone.
The "islets" were shown to have morphological integrity on histology, with a final purity of 10-20%. Viability was tested by in vitro insulin release studies.

Hesse et al (1989) attempted to isolate porcine islets using a method again based on the canine model of Horaguchi (1981). No information was provided on quantitative and qualitative assessment of the islet preparations. The preparations, which were unpurified, were autotransplanted and the high incidence of complications that ensued served only to confirm previous reports of the deleterious effects of exocrine contamination on graft function (Gray 1986, 1987). These occurred even in the absence of immunological problems, as in the autograft model.

A rather novel method was described by Calafiore in 1990. Pancreata were removed from slaughter-house pigs immediately at the time of death, with minimal warm ischaemia. The pancreas was distended with a collagenase and elastase solution, after which it was continually perfused with the same solution at 37°C in a water bath. After thirty minutes, the pancreas and water bath were shaken, whereupon the pancreas fell apart. After chilling the preparation with cold Hanks solution, islets were found free floating in the supernatant, with exocrine tissue and undigested pancreas falling to the bottom in a tissue pellet. After aspiration of successive aliquots of supernatant, further purification was performed on Ficoll density gradients. Dithizone was used to identify islets. Only islets with a diameter >60μm were included in counts. A final yield averaging over 11 000 islets per gram pancreas was obtained, with a purity of 95%. Viability was shown by fluorescent staining methods, in vitro insulin release and encapsulation and transplantation into NOD mice and Lewis rats. These remarkable results have yet to be repeated elsewhere; they may represent an effect of the local donor animal.

A similar method was described by Yamaguchi et al in 1992 as a means of isolating the islets of young (<6 months, <35 kg) pigs. After intraductal collagenase distension, the gland was gently shaken in an Erlenmeyer flask containing more collagenase solution, and islets were collected in the supernatant. The digest was subjected to discontinuous BSA density gradient centrifugation with a mean yield of 4000 islets per gram. Out of twenty four preparations, islet equivalent figures were only given for six, suggesting that significant islet fragmentation had occurred. Reasonable in vitro insulin release was shown but insulin independence could not be produced in a pancreatectomized pig.

Marchetti et al (1991) and Finke et al (1991) combined automated digestion with large-scale Euro-Ficoll density gradient purification. Islet yield in the digest was
approximately 5000 per gram pancreas (3000 Islet Equivalents per gram pancreas, using 150μm diameter islet equivalents). After purification, yield was approximately 4000 islets and 2500 IE per gram pancreas, with 4.4mm³ islet tissue per gram pancreas. Purity exceeded 90%. Excellent viability was shown in perifusion studies and on transplantation of 1000 islets into diabetic mice. Marchetti and Swanson (1992) cultured porcine islets for twenty one days in CMRL 1066 with 10% fetal calf serum and antibiotics. The islets maintained viability as shown on in vitro and in vivo testing, and also expressed significantly less class II antigens after culture.

A major advance in the isolation of human islets was the development of an “automated” method of pancreatic digestion (Ricordi 1989). This method was subsequently adapted to the isolation of porcine islets (Ricordi 1990). The automated method employs the use of a digestion chamber, in which the pancreas (already distended with collagenase) is surrounded by continually flowing tissue medium and simultaneously agitated by a shaking device to which it is attached. Once liberated, islets are immediately collected and protected from overdigestion or further mechanical trauma. As a concession to the fragility of porcine islets, minimal shaking was used as the pancreas digested. Slaughter-house pigs were again used as donors, but the pigs used were significantly older (1-3 years versus 6-8 months) and warm ischaemia was virtually nil, with the pancreas being removed at the moment of death. Islets were purified on Ficoll density gradients and identified with dithizone. In the digest, islet yield averaged 10 000 islets per gram pancreas, and after purification, final yield averaged 4 000 islets per gram with a purity of 80-95%. Diabetes in six out of seven diabetic nude mice was reversed with return of diabetes after nephrectomy.

This is probably the most reproducible and useful method developed to date. The important elements in successful porcine islet isolation seem to be:
1. The use of an effective enzyme.
2. The avoidance of overdigestion.
3. The avoidance of unnecessary mechanical trauma.

Other important factors may well include the minimizing of warm ischaemia (Hering 1989, Ricordi 1990) and the use of older pigs as donors, as the islets tend to have a better developed connective tissue capsule (Hering 1989) protecting them from the adverse effects of collagenase and trauma. Further advances have been described in the purification stage, notably with the use of Euroficoll gradients (Finke 1991), but the single most important stage certainly appears to be an effective, gentle initial pancreatic digestion.
A major problem in the use of pig islets for transplantation is the preservation of the islets before actual transplantation. Porcine islets are very difficult to culture and lose glucose responsiveness in vitro with the usual culture media. As porcine islets are being studied as human analogues, it seems logical to examine the effect of human serum in the culture medium. Human serum has provided the best culture medium for fetal porcine islet-like cell clusters (Korsgren 1988) and RPMI 1640 with 10% human serum has recently been shown to maintain porcine islet viability for up to 44 days (Hill 1992).
Figs 3.1 and 3.2  The Molecular Structures of Human and Porcine Insulin
Fig 3.3  Section of Porcine Pancreas (Large White / Welsh Cross. Standard Abbatoir Pig). Mag x50. H&E stained.

Fig 3.4  Section as above, stained for insulin.
Fig 3.5  Section of Porcine Pancreas (Large White / Welsh Cross. Standard Abbatoir Pig). Mag x100. H&E stained.

Fig 3.6  Section as above, stained for insulin.
Fig 3.7  Porcine Islet x200. Note vascular channel and loosely packed cellular structure.

Fig 3.8  Islet as above, stained for insulin.
CHAPTER 4

THE COLLAGENASE DIGESTION OF THE PORCINE PANCREAS

4.1 Introduction

4.2 Methods
   - Pancreatic Tissue
   - Reagents
   - Digestion Method
   - Islet Quantification
   - Density Gradient Purification

4.3 Results

4.4 Discussion

4.5 Figures & Tables
4.1 Introduction

The basic principle behind islet isolation is the dissociation of the pancreas by the enzyme collagenase into a cell suspension, (the digest) from which islets can then be purified. Collagenase digestion is the standard method of releasing islets from the pancreata of all species; and intraductal delivery of the enzyme can produce large volumes of digest containing islets and dispersed exocrine tissue (Horaguchi 1981, Gray 1984).

The specific advance described by Gray was the administration of collagenase via the pancreatic duct. This results in its distribution around the acini of the gland, such that after incubation the exocrine tissue becomes separated from the islets. This digestion stage is critical, because if the pancreas is over-digested the islets are broken down into single cells, whilst if the pancreas is under-digested the islets are surrounded by a fringe of exocrine tissue which greatly impairs subsequent purification. Islet purification is dependent on the effective separation of the islets from the exocrine and this is only possible if the islets are lying free, well cleaved from the exocrine, in the initial pancreatic digest.

Porcine islets are much more irregular in shape than human or rat islets (see Chapter 3), containing wide vascular channels rendering them quite fragile and susceptible to fragmentation by overdigestion or trauma (Ricordi 1986). Thus an effective method of porcine pancreatic digestion depends on the prevention of overexposure of the islets to the action of collagenase. This study describes a method of porcine pancreatic digestion designed to prevent this overexposure. This method has not previously been used for the digestion of the porcine pancreas.

The most frequently used method of islet purification uses density gradient centrifugation, relying on the intrinsic density of different cells or tissue to achieve separation. Ficoll, a polymer of sucrose is the most commonly used gradient medium, but has been shown to detrimentally affect islet viability (Lake 1987). Bovine serum albumin (BSA) has particular advantages for density gradient separation because solutions have a low viscosity, a general protective effect on cells and induce less cellular aggregation (Shortman 1972). Also, high density BSA solutions can be produced with constant osmolality. For these reasons, BSA was chosen as the density gradient medium for islet purification. BSA has not previously been used to isolate porcine islets. The criteria of: 1) Quantification of the islets in the digest, and 2) Purity
of the islets after BSA density gradient centrifugation were used to assess the results of these techniques.

4.2 Methods

Pancreatic Tissue
Porcine pancreata were obtained from a local abattoir with a median warm ischaemia time of seven minutes (range five to nine minutes). Donor pigs were Large White / Welsh cross breed, the standard British "pork" pig, 6-8 months old, with an average weight of 80-100kg. The pancreas was removed and transported to the laboratory in hyperosmolar citrate (Travenol. Thetford, UK) at 4°C, on ice, in a sterile plastic bag.

After removal of fat and lymphoid tissue in the laboratory, the pancreatic mass was recorded and the duct cannulated, (Fig 4.1), with a size 3 FG catheter (Portex, Hythe, Kent, UK). The catheter was ligated in place and ligatures were also placed on either side of the 'middle' lobe in order to isolate and selectively distend the splenic lobe.

Reagents
Collagenase
Powdered collagenase was obtained from the Serva company, Heidelberg, FRG. Type 17449, was used throughout. This was dissolved in Hanks Balanced Salt Solution (HBSS) from Flow laboratories, Rickmansworth, UK, a process aided by ultrasonic dispersion. Final volumes of solution contained collagenase at a concentration of 2mg/ml and a 10% concentration of the enzyme DNA-ase (Sigma Chemical Co Ltd, Poole, UK). Prepared collagenase solution was then stored at -20°C until required.
Minimal Essential Medium
Sterile bottles of Minimal Essential Medium (Flow Labs Ltd, Herts, UK) were used as the main tissue medium throughout the study, as improved viability (judged by Trypan Blue exclusion) was observed in islets maintained in this medium over a period of time when compared with Hanks Balanced Salts Solution. The medium was prepared by dilution of concentrated MEM (10X) with sterile water and supplemented with Streptomycin (100 U/ml), Penicillin (100 U/ml) and Amphoterecin (2.5 µg/ml).

New-Born Calf Serum
Fetal or new-born calf serum (Advanced Protein Products Ltd, Brierley Hill, UK) was added in varying concentrations to prevent tissue adhering to plastic tubing and vessels.

Dithizone
Diphenylethylcarbazone (Dithizone) was used throughout for the identification of islets. This substance binds to the zinc in the insulin molecule, staining it a vivid red (Latif, 1988) enabling direct identification on microscopy. Dithizone was obtained from the Sigma Chemical Co. and made up to 1.2 mM in 5% (v/v) dimethylsulphoxide (DMSO) / MEM / 1% BSA.

Bovine Serum Albumin
Stock solutions of 35% BSA were obtained from Advanced Protein Products Ltd, Brierley Hill, UK. The BSA was stored at -20°C until needed and then thawed at room temperature. The osmolality of BSA is 300 mOsmol/kg, virtually identical to that of porcine serum (305 mOsmol/kg). Differing densities of BSA were achieved by dilution with MEM and the densities measured and adjusted by means of a digital densitometer (DMA 35, Parr Scientific Ltd, London, UK).

Digestion Method
The pancreas was warmed for 2 minutes in MEM at 35°C and then distended by the injection into the cannula of the collagenase, 2 ml of collagenase per gram of pancreas, at pH 7.6 and 35°C. Progressive uniform distension (Fig 4.2) was obtained by the gradual withdrawal of the cannula during injection.

A biopsy was taken from the region of the pancreas that was seen to distend first, usually the tail (Fig 4.3), and divided into five pieces. Each piece was incubated at 35°C in a Universal container with 1 ml pre-warmed dithizone. The pancreas was similarly incubated in MEM at 35°C. At five minutes of incubation, a single container
was removed from the water bath, shaken for thirty seconds and the contents examined by incident white-light microscopy. If a single cleaved (totally free of exocrine tissue) islet was seen (Fig 4.4), the pancreas was removed from incubation. If no cleaved islets were present (Fig 4.5), further biopsies were examined at one minute intervals until cleaved islets were present.

When cleaved islets were seen, the pancreas was removed from the incubation medium and placed into one half of a kidney bowl divided by a 1mm mesh, and gently teased apart with forceps. The liquid digest that passed through the mesh was aspirated and passed through a 500μm mesh into MEM at 4°C containing 20% NBCS and 0.004mg/ml DNAase. A further 50 ml of MEM at 35°C containing 10% NBCS was added to the kidney bowl, and the teasing and aspiration continued until digestion ceased, usually at 10-30 minutes. Digestion was judged to have ceased when no further dispersal of the remaining pancreatic tissue occurred despite teasing.

The total amount of liquid digest (Fig 4.6) produced, was washed three times by centrifugation at 100G for 2 minutes in MEM with 10% NBCS. The resulting final digest was pooled and resuspended in MEM with 10% NBCS in a 50ml conical flask.

Samples were taken for islet quantification.

Islet Quantification
After inversion and dispersal, 5 X 200μl samples were taken from the 50ml flask containing the final digest volume, Fig 4.7. Each was diluted ten times with MEM and two 100μl samples from each then placed on a Petri dish, to give a total of ten samples. Dithizone (100μl, diluted to one tenth concentration) was added and each sample examined microscopically on a Nikon microscope at 10 X magnification with white light side illumination against a dark background.

Islet Numbers
The total number of islets >50μm diameter was counted in each sample. The total islet count was calculated from the sample mean:

\[
\text{Dilution Factor of 100μl sample from 50ml total volume} = 5000
\]

Mean Count from 10 samples X 5000 = Total No. Islets

\[
\text{No. Islets / gram pancreas} = \frac{\text{Total No. Islets}}{\text{Mass of Pancreas}}
\]
Mean Islet Volumes

The mean islet volume was calculated by measuring 100 consecutive islet diameters in the dithizone stained samples using a pre-calibrated eyepiece graticule micrometer at x40 magnification. The volume \((0.524 \times d^3)\) of each islet was computed and the mean islet volume calculated:

- Islet diameter on graticule scale \(\times 0.01 = \text{Islet Diameter in mm.}\)
- Islet Volume \((\text{mm}^3) = \text{Islet Diameter}^3 \times 0.524\).

This was calculated for each islet diameter, 100 consecutive diameters in each preparation, the mean volume being calculated from the mean of 100 volumes and not from the mean islet diameter.

Islet Tissue per gram Pancreas

The total amount of islet tissue produced in the digest per gram of original pancreatic mass was calculated:

\[
\text{Islet Tissue /g} = \text{No. Islets/g} \times \text{Mean Islet Volume}
\]

Islet Equivalents

Islet equivalent calculations were made using a mean islet diameter of 105µm, the mean histological islet diameter of the porcine pancreata used throughout these studies.

Volume of a 105µm diameter islet = \(6.06 \times 10^{-4}\text{mm}^3\).

\[
\text{No. Islet Equivs / gram} = \frac{\text{Islet Tissue /g}}{6.06 \times 10^{-4}}.
\]

Density Gradient Purification

The possibility of purifying the islets of each preparation was investigated by placing a sample of each onto a continuous BSA density gradient. After centrifugation, the amount of islet / exocrine separation was determined by insulin and amylase assays of aliquots down the gradient.
With each half-pancreas digested, two 1ml aliquots of the 50ml digest tube were suspended in 1ml of high density (1.095g/cm$^3$) BSA and overlain with BSA from a gradient maker, containing in its chambers BSA of densities 1.059g/cm$^3$ (low density) and 1.077g/cm$^3$ (high density), Fig 4.8. The osmolality of the BSA in all cases was that of porcine serum, 300 mOsmol/kg.

The resulting tube contained BSA of gradually decreasing density from the bottom to the top, with the aliquot of digest lying at the bottom. Two gradients from each preparation were centrifuged at 500G for 20 minutes at room temperature and eleven 1ml aliquots removed from each, sequentially down the gradient. Each aliquot was washed twice in MEM/10% NBCS, sonicated for two minutes to disrupt all tissue and divided for insulin and amylase assay.

**Insulin Assay**
Insulin was extracted with 5 ml acid / alcohol (H$_2$SO$_4$ / C$_2$H$_5$OH, 1:40 v:v) at 4°C for 24 hours. Following dilution with phosphate buffer the insulin content was measured using radioimmunoassay (see Appendix 1).

**Amylase Assay**
Amylase assay was performed using the hydrolysis by the amylase of a water-insoluble starch polymer carrying a blue dye to form water-soluble blue fragments (see Appendix 2).

The percentage density distribution of insulin (islets) and amylase (exocrine), could thus be quantified. For each gradient a graph was constructed, plotting absolute and cumulative insulin percentage, and cumulative amylase percentage, all against density.
4.3 Results
Ten consecutive porcine pancreatic splenic lobes underwent collagenase digestion as described above. All values are median (range).

Digest Islet Quantification
66g (47g-97g) of porcine pancreas was distended with 2ml/g of Serva collagenase (2mg/ml). As the distended pancreata were incubated, simultaneously incubated biopsies revealed the presence of cleaved islets at 14 (7-20) minutes.

After manual disruption of the digested pancreas, a digest volume of 5.5ml (2-19) was obtained.

Samples of the digest were stained with dithizone and examined microscopically (Fig 5.1). The digest contained 1 423 (400-2 902) islets per gram of original pancreas mass. Only islets with a diameter of 50μm or greater were counted. A cleavage index (% of islets completely free of surrounding exocrine tissue) of 79.2% (64%-92%) was obtained.

The mean diameter of islets (>50μm) in the digest was 79.2μm (62-106μm), considerably smaller than the mean histological diameter of islets from similar porcine pancreata. After the individual measurement of 100 consecutive islet volumes per preparation, a mean islet volume figure of 6.11 x 10^{-4}mm^3 (2.05-9.32 x 10^{-4}mm^3) was calculated.

Calculations using the above data showed that the amount of islet tissue / gram produced was 0.97mm^3 (0.26-2.10mm^3), and the number of 105μm diameter islet equivalents / gram was 1 312 (429-3465).

Full details of this data are given in Table 4.1.

Density Gradient Purification
Samples of the digest from the first seven of the ten preparations were placed onto continuous density gradients of BSA of porcine physiological osmolality. Density in the gradient ranged from 1.055g/cm^3 to 1.077g/cm^3.

The sequential 1ml aliquots down the gradient tube after centrifugation were aspirated and assayed for insulin and amylase levels. This enabled the construction of graphs
plotting the distribution of cumulative and absolute insulin and amylase levels, as given in Figs 4.9 - 4.15

The major index of purity was the percentage of the total amount of amylase present at the level in the gradient at which 70% of the total insulin could be isolated, i.e. % Amylase at 70% Insulin. A median value of 58% (19%-65%) was seen.

The index of efficacy of the density gradient in separating islet tissue from exocrine was the percentage of insulin remaining in the bottom 3 aliquots of the gradient, the "pellet", i.e. % Insulin in Pellet. A median value of 34% (8%-48%) was obtained.

Full details of the islet / exocrine separation can be seen from the graphs in Figs 4.9 - 4.15. Only the graphs of the first seven pancreatic preparations are given, as analyses were discontinued as the results of these first seven became available.

4.4 Discussion

Many methods have been described for islet isolation from the rodent (Lacy et al. 1967), canine (Warnock et al. 1989), bovine (Hering et al. 1989), primate (Gray 1986) and human pancreas (Gray 1984). Islet transplantation for the treatment of diabetes mellitus has been achieved in rodents, dogs and recently in humans (Scharp et al. 1991). The results with human islets indicate that successful transplantation will succeed only in the presence of an adequate viable islet cell mass, and thus one of the major problems is the production of an adequate yield of islets from a single donor pancreas. The other main problem is rejection. If islet transplantation becomes clinically applicable, meeting the requirement for islet tissue on the basis of human organ donation will be impossible. Pig islets could represent an optimal source of islet tissue because of the similarity between human and porcine insulin and because of the availability of pig pancreata.

The aim of these experiments was to attempt the collagenase digestion of the porcine pancreas, to produce a digest in which porcine islets were intact and capable of being separated from exocrine tissue. Previous studies into porcine islet isolation had revealed the fragility of porcine islets (Ricordi et al. 1986) due to their lack of capsule, and so a new method of isolation was tried (London et al. 1990) in an attempt to avoid the damaging effects of overdigestion.

After empirical studies to find an effective collagenase for the pig pancreas, ten consecutive pancreata were digested by the method described above, using SerVa
17449 collagenase. Reasonably effective digestion was produced, with a mean digest volume of 5.5ml but digestion was far from complete in many cases as seen by the cleavage index of only 79.2%, with many islets still being surrounded with significant rims of exocrine tissue. Also, a remnant of undigested pancreas, often representing 25-30% of the original pancreatic mass was usually left behind in the kidney dish after effective digestion had ceased.

The mean islet volume of $6.11 \times 10^{-4}\text{mm}^3$ resulted in a total amount of islet tissue per gram of pancreas of $0.97\text{mm}^3$, with 1312 islet equivalents per gram. The most significant result of the digest islet quantification was the small mean islet diameter, less than the mean histological diameter, and the low mean islet volume. This clearly showed that islets were still being broken up during this digestion method, almost certainly by collagenase overdigestion. Thus, this method seemed to be producing a rather ‘patchy’ digestion, with many islets remaining within the parenchyma of the pancreas, and the islets that are being released are being broken up, presumably by overexposure to the enzyme.

The reasons for using BSA density gradient centrifugation for the purification of porcine islets have been given in section 4.1. On isolating 70% of available insulin (islets) in a density gradient, a mean contaminating level of exocrine tissue of 58% was obtained. As exocrine tissue represents 98-99% of the total tissue in the pancreas, 58% of total exocrine tissue in a ‘purified’ islet preparation makes a nonsense of the term, this is unfortunately a completely unacceptable level of contamination. Similarly, a mean value of 34% of the total islet tissue left behind in the pellet after density gradient centrifugation cannot be seen as an acceptable value for an efficient method of islet isolation.

Viability testing on the islets preparations obtained during these studies were not performed, as the purity of the final preparation could not be expected to reverse diabetes.

In only a single preparation was an acceptable value of 20% contamination, with 2% unavailable insulin, obtained. This might indicate that acceptable figures can be obtained with collagenase digestion of the pig pancreas, but the means of improving the results and producing consistently acceptable purification remain to be determined.
Although the digestion method is not perfect, it does produce a small number of porcine islets, and as the availability of porcine pancreata is almost limitless, this may not prove a great problem. A greater problem lies in the effective separation of porcine islets. Manipulation of the properties of the density gradient can be performed in an attempt to improve the final preparation purity. The medium itself, BSA, has proved effective in the isolation of rodent (Lake et al 1987) and human islets (London et al 1990) and so it is reasonable to expect that it will ultimately be effective in the preparation of porcine islets. Certainly, the viability of islets has been shown to be superior in those isolated in BSA as opposed to Ficoll (Lake et al 1986).
Fig 4.1  The Porcine Pancreas Cannulated Prior to Distension.

Fig 4.2  The Porcine Pancreas Distending with Collagenase during Intraductal Injection
Fig 4.3 A Biopsy Specimen being taken from the Splenic Lobe of the Collagenase-Distended Porcine Pancreas

Fig 4.4 Biopsy Specimen x100, after Incubation, showing 'Cleaved' Islets.
Fig 4.5  Biopsy Specimen, x100, After Incubation, showing 'Non-Cleaved' Islets, still attached to Exocrine Tissue. Dithizone stained.

Fig 4.6  An Example of Porcine Pancreatic Digest after Collagenase Digestion. Magnification x50. Dithizone Stained.
Fig 4.7 Islet Quantification Procedure

- Total Digest to 50 ml
- 200 μl 200 μl 200 μl 200 μl 200 μl
- MEM 1.8 ml
- 100 μl + 100 μl Dithizone
- Microscopy
- Petri Dish
Fig 4.8  
**DENSITY GRADIENT CONSTRUCTION**

Low Density BSA  
High Density BSA  

PUMP  

Digest
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<th>Prep No.</th>
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<th>Mean Diameter (μm)</th>
<th>Cleavage Index</th>
<th>Islet Volume (mm³)</th>
<th>Tissue Equivs per gram</th>
<th>Mean Digest Islet Volume per gram</th>
<th>Mean Digest Islet Tissue per gram</th>
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Fig 4.9

300mOsmol BSA Separation
PREP 1

% Amylase at 70% Insulin: 58%
% Insulin in Pellet: 34%

Cumulative Insulin
Cumulative Amylase
Absolute Insulin

Fig 4.10

300mOsmol BSA Separation
PREP 2

% Amylase at 70% Insulin: 58%
% Insulin in Pellet: 36%

Cum Ins
Cum Amy
Abs Ins
Fig 4.11

300mOsmol BSA Separation
PREP 3

% Amylase at 70% Insulin: 61%
% Insulin in Pellet: 24%

DENSITY g/cm³

Cum Ins
Cum Amy
Abs Ins

Fig 4.12

300mOsmol BSA Separation
PREP 4

% Amylase at 70% Insulin: 19%
% Insulin in Pellet: 17%

DENSITY g/cm³

Cum Ins
Cum Amy
Abs Ins
Fig 4.13

300mOsmol BSA Separation
PREP 5

% Amylase at 70% Insulin: 65%
% Insulin in Pellet: 38%

Fig 4.14

300mOsmol BSA Separation
PREP 6

% Amylase at 70% Insulin: 31%
% Insulin in Pellet: 8%
Fig 4.15

300mOsmol BSA Separation
PREP 7

% Amylase at 70% Insulin: 61%

% Insulin in Pellet: 48%

Density g/cm³

Graph showing Cum Ins, Cum Amy, and Abs Ins vs. Density.
Fig 4.16

**% INSULIN IN PELLET**

After 7 consecutive digestions and 300mOsmol BSA gradient separation

- Median 34% (8-48)

Fig 4.17

**% AMYLASE AT 70% INSULIN**

After 7 consecutive digestions and 300mOsmol BSA gradient separation

- Median 58% (19-65)
CHAPTER 5

THE OPTIMIZATION OF ISLET PURIFICATION USING HYPEROSMOLAR DENSITY GRADIENT CENTRIFUGATION

5.1 Introduction.

5.2 Methods
Density Gradient Construction.

5.3 Results.

5.4 Discussion.

5.5 Figures & Tables.
§ 1. Introduction

The later stages of islet isolation require the purification of islets from the pancreatic digest. It is generally agreed that it is desirable to purify islet preparations, in order to reduce their immunogenicity and allow safe and successful transplantation (Gray 1984). This is currently best achieved by density gradient centrifugation (Brakke 1951). The technique relies on the fact that islets are less dense than exocrine tissue. Thus when the pancreatic digest is placed on to a density gradient, islets migrate to their area of buoyant density and are separated from exocrine tissue.

The only purification technique which has been successfully applied on a large scale is that of isopycnic density gradient centrifugation. The density medium most commonly used has been Ficoll although some groups, including ourselves (Lake 1989) have used bovine serum albumin (BSA). One of the advantages of using BSA rather than Ficoll for the construction of continuous gradients as described in this study, is that it is possible to construct pure density gradients without any change in osmolality (Williams 1972). This is not true of Ficoll; because of its water binding properties it produces a change in both density and osmolality in the more dense parts of the gradient (Williams 1972). BSA has a high molecular weight and therefore exerts a minimal osmotic effect, unlike Ficoll and Dextran it does not bind water at high concentrations. This means that it is possible to construct a continuous gradient of changing density but fixed osmolality. The viability of islets isolated in BSA is also superior to those isolated in Ficoll (Lake 1987).

Studies on human islet isolation in this laboratory indicate that the purity of human islet preparations varies markedly; this variation results from changing exocrine tissue density from one pancreas to another (Appendix 3). Electron micrographic studies confirm that exocrine tissue becomes less dense and approaches the density of islets due to exocrine enzyme discharge. These studies thus show that the variable purity of human islet preparations results mainly from a change in exocrine tissue density. The electron micrographic studies of exocrine tissue from the low and high density areas of continuous gradients showed that there was no difference in the viability of the exocrine tissue in the two areas (as judged by EM criteria), but that the exocrine tissue in the low density area, i.e. contaminating exocrine tissue, contained fewer exocrine granules. This would suggest that exocrine tissue becomes less dense due to the discharge of exocrine enzymes. It is probable therefore that islet purity could be improved; either by measures to minimise exocrine enzyme discharge, or techniques to increase the density of exocrine tissue relative to endocrine tissue.
The factors that could affect exocrine tissue enzyme discharge (and thus density) are protean, and include the nature of the donor’s death, warm ischaemia, cold ischaemia, the batch of collagenase used and the technique of pancreas digestion and density gradient centrifugation.

Investigations into the cause of an apparent batch to batch variation in the efficiency of BSA for the purification of human islets showed a marked variation in the osmolality of BSA from various sources (290-550 mOsm/kg water). It was also shown that the density of human islets increases with increasing osmolality of the BSA in which they are suspended. Experiments using computer analysed videotape recordings of the volume changes of islets and exocrine tissue in response to an increasingly hypertonic environment, showed that exocrine tissue increased in density relatively more than islets (Taylor 1990) - resulting in an improved purity in the hyperosmolar BSA gradients (Appendix 4). This results from the fact that a hyperosmolar environment differentially affects exocrine and endocrine tissue. More water would appear to be drawn out of exocrine tissue relative to endocrine and therefore the exocrine tissue increases in density relatively more than the endocrine. In conclusion, we have found that hypertonic density gradients yield human islets of greater purity than isotonic gradients.

In summary therefore the background to this study is that the variable purity of human islet isolations results from the discharge of exocrine enzymes. If islets of a high purity are to be produced on a regular basis then techniques must be developed for either minimizing this exocrine enzyme discharge or increasing the density of exocrine tissue relative to the islets. Manipulation of the osmolality of the isolating medium has a strikingly beneficial effect on increasing the relative density of exocrine tissue with resulting increase in islet purity.

The effect of a hyperosmolar gradient medium has never been tested on the isolation of porcine islets. Thus, as a result of the dissatisfying islet purities obtained with isomolar BSA density gradient centrifugation (Chapter 4), a test of this effect was performed.
5.2 Methods

Pancreatic Tissue
Porcine pancreata were obtained and prepared as described in Chapter Four.

Digestion Method
Porcine pancreatic digest was prepared from seven consecutive pancreata by the manual digestion technique as described in Chapter Four.

Reagents
MEM, NBCS, and collagenase was prepared as described in Chapter Four.

BSA
Bovine serum albumin was obtained as 35% stock solution from Advanced Protein Products (APP) Ltd, Brierley Hill, UK. Osmolalities of 300mOsm/kg, 400mOsm/kg and 500mOsm/kg were specially prepared by APP Ltd.

Densities were altered by dilution with MEM of corresponding osmolalities and measured and adjusted with a digital densitometer (DMA 35, Parr Scientific Ltd, London, UK).

Seven consecutive pancreata were digested. For each preparation, a sample of the digest was subject to separation on continuous density gradients as described in Chapter Four. Samples from each preparation were placed, in duplicate, on density gradients of the three differing osmolalities:
Islet Separation on Hyperosmolar Density Gradients

Density gradients were constructed using the gradient maker as described in Chapter Four.

300 mOsmol Gradient
Three aliquots of digest were suspended in 1ml BSA of density 1.077 g/cm³ (osmolality 300mOsm/kg H₂O, measured by vapour pressure osmometry), and overlaid with 10ml BSA from a continuous gradient maker containing BSA of density 1.059 and 1.077g/cm³ (both 300 mOsm/kg H₂O). This produced a density gradient ranging from 1.059 (fraction number one) to 1.077 g/cm³ (fraction number eleven). The osmolality was 290 mOsm/kg H₂O throughout the gradient. The gradients were then centrifuged at 500g for twenty minutes and eleven 1 ml aliquots (fraction numbers 1-11) removed sequentially from each gradient.

The aliquots were extracted for insulin and amylase content as described in Appendices 1 & 2. The insulin and amylase content of each fraction was expressed as a percentage of the total insulin and amylase present in the gradient, and the mean value calculated using the two gradients from each osmolality gradient from each isolation. With each isolation it was therefore possible to calculate both the 'peak density' of insulin and amylase and also their cumulative percentage distribution for a 300mOsm/kg BSA gradient.
400 mOsmol Gradient
Three aliquots of digest were suspended in 1 ml BSA of density 1.092 g/cm³ (osmolality 400 mOsm/kg H₂O, measured by vapour pressure osmometry) and overlaid with 10 ml BSA from a continuous gradient maker containing BSA of density 1.065 and 1.092 g/cm³ (both 300 mOsm/kg H₂O). This produced a density gradient ranging from 1.065 (fraction number one) to 1.092 g/cm³ (fraction number eleven). The osmolality was 400 mOsm/kg H₂O throughout the gradient. The gradients were then centrifuged and the aliquots removed and extracted for insulin and amylase as described above.

The insulin and amylase content of each fraction was expressed as a percentage of the total insulin and amylase present in the gradient and the mean value calculated using the two gradients from each osmolality gradient from each isolation. With each isolation therefore it was possible to calculate both the 'peak density' of insulin and amylase and also their cumulative percentage distribution, for a 400 mOsm/kg BSA gradient.

500 mOsmol Gradient
Three aliquots of digest were suspended in 1 ml BSA of density 1.095 g/cm³ (osmolality 500 mOsm/kg H₂O, measured by vapour pressure osmometry) and overlaid with 10 ml BSA from a continuous gradient maker containing BSA of density 1.065 and 1.095 g/cm³ (both 500 mOsm/kg H₂O). This produced a density gradient ranging from 1.065 (fraction number one) to 1.095 g/cm³ (fraction number eleven). The osmolality was 500 mOsm/kg H₂O throughout the gradient. The gradients were then centrifuged and the aliquots removed and extracted for insulin and amylase as described above.

The insulin and amylase content of each fraction was expressed as a percentage of the total insulin and amylase present in the gradient and the mean value calculated using the two gradients from each osmolality gradient from each isolation. With each isolation therefore it was possible to calculate both the 'peak density' of insulin and amylase and also their cumulative percentage distribution, for a 500 mOsm/kg BSA gradient.
6.3 Results

All values are median (range).

During the course of seven consecutive porcine pancreatic digestions, a median pancreatic mass of 59g (50g-63g) was digested, giving a digest volume of 5.0ml (3ml-9ml), with fully cleaved islets representing 78% (62%-91%) of the total.

Islet quantification of the digest as in the previous chapter gave median values of:

- Islets per gram pancreas: 2,074 (1,532 - 2,960)
- Mean Islet Diameter: 97μm (84 - 103)
- Mean Islet Volume: 6.2 x 10^-4mm³ (3.4 - 10.5)
- Islet Tissue /g: 1.39mm³ (0.52 - 2.52)
- Islet Equivalents /g: 2.293 (0.52 - 4.158)

Full details of this data are given in Table 5.1.

Samples of the digest from each preparation were placed onto continuous density gradients of the three different osmolalities as described above. After centrifugation, aliquots were aspirated and assayed as described. Graphs were constructed (Figures 5.1 - 5.21) as in the previous chapter. The absolute values of

1) % amylase at 70% insulin
2) % insulin in pellet,
were obtained from these graphs and are given in Tables 5.2 and 5.3.

Non-parametric statistical testing of the values in Tables 5.2 and 5.3 showed a significant difference (p <0.01) between the values obtained after 400mOsmol BSA density gradient centrifugation and those of both other osmolalities. The medians of these values are:
% Amylase at 70% Insulin

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<th>Osmolality</th>
<th>Amylase (%)</th>
</tr>
</thead>
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<td>300mOsmol/kg</td>
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</tr>
<tr>
<td>400mOsmol/kg</td>
<td>28% (2.5 - 45%)</td>
</tr>
<tr>
<td>500mOsmol/kg</td>
<td>48% (15 - 76%)</td>
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</tbody>
</table>

% Insulin in Pellet

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<tr>
<th>Osmolality</th>
<th>Insulin (%)</th>
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</thead>
<tbody>
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<td>300mOsmol/kg</td>
<td>48% (10 - 73%)</td>
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<td>400mOsmol/kg</td>
<td>23% (7 - 41%)</td>
</tr>
<tr>
<td>500mOsmol/kg</td>
<td>68% (25 - 70%)</td>
</tr>
</tbody>
</table>

The difference is illustrated in the graphs, (Fig 5.22 & 5.23,) plotting the median values for the three osmolalities.
The use of hyperosmolar BSA at 400mosmol/kg in a density gradient produces a significant improvement in the purity of isolated porcine islets of Langerhans.

5.4 Discussion

Islets are more difficult to obtain in pure form from the large, fibrous mammalian pancreas than from the rodent pancreas. It is however necessary to obtain pure islets as there is evidence to suggest that exocrine contamination of collagenase-prepared islets results in impaired implantation to a transplantation site (Hesse et al 1986, Gray et al 1986), and also maximizes the immunogenic response to islet transplants, with resulting rejection (Gotoh et al 1986). More importantly, unpurified islet preparations produce disastrous complications such as portal hypertension and disseminated intravascular coagulation when injected intraportally (Mehigan et al 1981).

The eventual aim must be to transplant 100% pure islet tissue with no loss of yield, especially in human islet transplantation where the donor organ situation is so critical.
The use of a purification process after pancreatic digestion inevitably causes a reduction in islet yield; a trade-off between yield and purity. Some compromise of yield in order to obtain a 'reasonable' degree of purification is necessary for safe and effective transplantation.

Although Ficoll density gradient separation remains the standard method of islet purification, work from this laboratory has shown it to have a deleterious effect on islet
viability when compared to BSA (Lake et al 1986). Other work has shown no difference in viability between BSA-isolated and hand-picked human islets (Lake et al 1989). Initial experiments using isosmolar BSA for porcine islet purification did not give islet preparations of satisfactory purity (See Chapter Four). It is well established that the density gradient separation of certain cell types is influenced by gradient medium osmolality. Boyum (1983) demonstrated that the purification of monocytes from human blood on Nycodein gradients was strongly influenced by osmolality. Blood monocytes are generally less dense than lymphocytes but some overlap occurs, a situation analogous to pancreatic endocrine and exocrine tissue. A similar phenomenon has been noticed for the isolation of rat and human natural killer cells on Percoll gradients (Timonen 1982) and for the separation of antibody-forming cells from spleen suspensions on BSA gradients (Williams 1972). Studies on human islets in this laboratory at the same time were showing that the use of hyperosmolar BSA was having a dramatic effect on the purity of human islet preparations. Experiments were showing (Taylor 1990) that with the use of hypertonic BSA at twice isotonic strength, a density difference between islets and exocrine 30% greater than with isotonic was produced. A maximum density difference of 35% could be produced with BSA at 3.09 times isotonic strength. The use of twice isotonic strength BSA was adopted for optimum separation without the imposition of undue hypertonic stress.

The effect of hyperosmolar BSA on porcine islet purification was studied in parallel with the above studies. Porcine islets are considerably more fragile than human islets (Ricordi et al 1986) and thus more at risk from hypertonic stress. With physiological porcine tonicity at 299-300mOsmol /kg, comparative experiments as described were set up at 300, 400 and 500mOsm /kg.

As expected the results of the digest quantification were similar to those produced in the previous chapter, with a mean islet volume of 6.2 x 10^{-4} \text{mm}^3, and total islet tissue per gram pancreas of 1.39mm^3.

A striking difference could be seen between the purity of islet preparations isolated in 400mOsmol /kg BSA and those isolated in 300 or 500mOsmol /kg BSA, as illustrated in Figures 5.22 & 5.23. The resulting mean contaminating level of 25% of total amylase and amount of unavailable insulin, 23%, differed significantly from the values obtained with the other osmolalities. Islets and exocrine tissue behave as osmometers in hyperosmolar BSA, the increased toxicity of the medium resulting in loss of water from the tissue with resulting increased density. It would seem that hyperosmolar BSA at 400mOsmol /kg has a more pronounced effect on the exocrine tissue, making it more
dense and dragging it down further in the gradient tube, away from the islets towards the top of the tube. At 500mOsmol/kg the tonicity of the BSA affects both tissues equally, dragging them down towards the bottom of the tube without separation.

It is interesting to note that recent reports of improved human and porcine islet isolation have involved the use of hyperosmolar density gradient media. Dialyzed Ficoll (Scharp et al 1973) is hyperosmolar and Euro-Ficoll solution (Ricordi et al 1991, Finke et al 1991, Olack et al 1991) has an osmolality of 450mOsmol /kg. It has been suggested that the improving effect on islet purity of Euro-Ficoll gradients is due to stabilization of the islet and exocrine tissues, as shown by good results even in sub-optimally perfused glands. The Euro-Ficoll may prevent further degeneration of exocrine cells, preventing enzyme discharge and thus preserving their high cell density relative to islets, but no evidence exists as yet to prove or disprove this theory.

The viability of human islets isolated in hyperosmolar BSA has been proven (Lake et al 1989, London et al 1990), as has the viability of human and porcine islets isolated in Euro-Ficoll (Finke et al 1991, Ricordi et al 1991). The viability of porcine islets isolated in 400mOsmol/kg BSA will be the subject of studies described in a later chapter.
Fig 5.1

300 mOsmol BSA Separation Prep 1

% Amylase at 70% Insulin: 15%
% Insulin in Pellet: 10%

Fig 5.2

400mOsmol BSA Separation Prep 1

% Amylase at 70% Insulin: 10%
% Insulin in Pellet: 15%
Fig 5.3

500mOsmol BSA Separation
Prep 1

% Amylase at 70% Insulin: 18%
% Insulin in Pellet: 25%

% Absolute Insulin
Cumulative Insulin
Cumulative Amylase

BSA Density g/cm³
Fig 5.4

300mOsmol BSA Separation
Prep 2

% Amylase at 70% Insulin: 52%
% Insulin in Pellet: 37%

Fig 5.5

400mOsmol BSA Separation
Prep 2

% Amylase at 70% Insulin: 35%
% Insulin in Pellet: 26%
Fig 5.6

500mOsmol BSA Separation
Prep 2

% Amylase at 70% Insulin: 35%
% Insulin in Pellet: 65%

Density g/cm\(^3\)

- Absolute Insulin
- Cumulative Insulin
- Cumulative Amylase
**Fig 5.7**

300mOsmol BSA Separation
Prep 3

% Amylase at 70% Insulin: 75%
% Insulin in Pellet: 50%

![Graph showing BSA density vs % Amylase and % Insulin](image)

- Absolute Insulin
- Cumulative Insulin
- Cumulative Amylase

**Fig 5.8**

400mOsmol BSA Separation
Prep 3

% Amylase at 70% Insulin: 45%
% Insulin in Pellet: 41%

![Graph showing BSA density vs % Amylase and % Insulin](image)

- Absolute Insulin
- Cumulative Insulin
- Cumulative Amylase
Fig 5.9

500mOsmol BSA Separation
Prep 3

% Amylase at 70% Insulin: 76%
% Insulin in Pellet: 67%

BSA Density g/cm³

- Absolute Insulin
- Cumulative Insulin
- Cumulative Amylase
**Fig 5.10**

300mOsmol BSA Separation
Prep 4

- % Amylase at 70% Insulin: 75%
- % Insulin in Pellet: 73%

**Fig 5.11**

400mOsmol BSA Separation
Prep 4

- % Amylase at 70% Insulin: 25%
- % Insulin in Pellet: 23%
Fig 5.12

500mOsmol BSA Separation
Prep 4

% Amylase at 70% Insulin: 15%
% Insulin in Pellet: 27%

Absolute Insulin
Cumulative Insulin
Cumulative Amylase
**Fig 5.13**

**300mOsmol BSA Separation Prep 5**

- % Amylase at 70% Insulin: 42%
- % Insulin in Pellet: 43%

![Graph showing BSA density and cumulative distribution of amylose and insulin](attachment:image1)

**Fig 5.14**

**400mOsmol BSA Separation Prep 5**

- % Amylase at 70% Insulin: 2.5%
- % Insulin in Pellet: 7%

![Graph showing BSA density and cumulative distribution of amylose and insulin](attachment:image2)
Fig 5.15

500mOsmol BSA Separation
Prep 5

% Amylase at 70% Insulin: 76%
% Insulin in Pellet: 68%

- Absolute Insulin
- Cumulative Insulin
- Cumulative Amylase

BSA Density g/cm³
Fig 5.16

300mOsmol BSA Separation
Prep 6

% Amylase at 70% Insulin: 56
% Insulin in Pellet: 48%

Fig 5.17

400mOsmol BSA Separation
Prep 6

% Amylase at 70% Insulin: 19%
% Insulin in Pellet: 26%
500mOsmol BSA Separation
Prep 6

% Amylase at 70% Insulin: 48%
% Insulin in Pellet: 53%
Fig 5.19

300mOsmol BSA Separation
Prep 7

% Amylase at 70% Insulin: 42%
% Insulin in Pellet: 56%

% 120
100
80
60
40
20
0

Absoute Insulin
Cumulative Insulin
Cumulative Amylase

BSA Density g/cm³

1.059 1.069 1.079

Fig 5.20

400mOsmol BSA Separation
Prep 7

% Amylase at 70% Insulin: 28%
% Insulin in Pellet: 19%

% 120
100
80
60
40
20
0

Absoute Insulin
Cumulative Insulin
Cumulative Amylase

BSA Density g/cm³

1.06 1.07 1.08 1.09 1.10
Fig 5.21

500mOsmol BSA Separation
Prep 7

% Amylase at 70% Insulin: 63%
% Insulin in Pellet: 70%

Absolute Insulin
Cumulative Insulin
Cumulative Amylase

BSA Density g/cm³
Fig 5.22

CONTAMINATING % AMYLASE AT 70% INSULIN

Median values after 7 consecutive preparations

Fig 5.23

% INSULIN REMAINING IN PELLET

Median values after 7 consecutive preparations
Table 5.1

CUMULATIVE DIGEST ISLET QUANTIFICATION

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<th>Prep No.</th>
<th>Mass Digest Volume g</th>
<th>Digest Islets No.</th>
<th>Mean Diameter μm</th>
<th>Cleavage Index %</th>
<th>Islet Volume x10^-4 mm³</th>
<th>Islet Tissue per gram</th>
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### Table 5.2

**% AMYLASE AT 70% INSULIN**

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### Table 5.3

**% INSULIN IN PELLET**

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CHAPTER 6

A PROSPECTIVE COMPARISON OF MANUAL AND AUTOMATED METHODS OF PORCINE PANCREATIC COLLAGENASE DIGESTION

6.1 Introduction

6.2 Materials & Methods

6.3 Results

6.4 Discussion

6.5 Figures and Tables
6.1 Introduction

The transplantation of human islets of Langerhans is rapidly becoming a realistic therapeutic option in the treatment of type 1 diabetes (Tzakis, 1990). The isolation of large numbers of viable islets from the pancreas is a prerequisite for successful transplantation. Efficient isolation depends on the dissociation of the pancreas into its separate components; this is usually accomplished by enzymatic collagenase digestion of the fibrous stroma followed by mechanical dispersion of the digested tissue (Gray 1987). The subsequent purification of islets from contaminating acinar tissue is usually achieved by density gradient centrifugation, and is critically dependent on the efficiency of the initial digestion process.

Although the delivery of collagenase by the intraductal route is now a standard technique for the enzymatic digestion of pancreata from humans and large animals (Gray 1984), many methods of mechanical dispersion have been employed, including manual shaking, teasing with forceps (Gray 1984, London 1990), mincing or maceration (Scharp 1989, Ricordi 1986, Alderson 1987), and trituration (Wamock, 1988, Sutherland 1974, Marchetti 1988). The most effective procedures depend on minimal mechanical trauma to the islets and their immediate removal, once liberated, from the injurious effect of collagenase.

Although two recently described techniques (London 1990, Ricordi 1988) fulfil these criteria and have been reported to produce similar results in the human, they have not yet been compared directly. The purpose of this prospective study was therefore to compare the results of porcine pancreatic collagenase digestion, using the automated method described by Ricordi et al, with those of the manual biopsy-based technique of London et al.

6.2 Materials and Methods

Pancreas Procurement

Porcine pancreata were obtained from a local abattoir with a median warm ischaemia time of seven (range 5-9) minutes. Donor pigs were of Large White – Welsh crossbreed, 6-8 months old, in the weight range 80-100kg. The splenic lobe of the pancreas was removed and transported to the laboratory in cold (4°C) hyperosmolar citrate solution (Travenol. Laboratories, Thetford, UK).
In the laboratory the gland was cleaned of fat and lymphoid tissue, weighed, and the main pancreatic duct cannulated with a 3-Fr catheter (Portex, Hythe, UK) which was ligated in place.

The pancreas was warmed for two minutes in MEM at 35°C and then distended (2ml/g pancreas) by injection via the cannula of Hanks balanced salt solution (pH 7.6, 35°C), containing 2mg/ml collagenase (Collagenase Pan Plus; Serva, Heidelberg, Germany) and 0.02mg/ml DNAase (Sigma Chemicals, Poole, UK). Uniform distension was obtained by the gradual withdrawal of the cannula during injection.

The distended pancreas was reweighed, sectioned longitudinally (Figs 6.1 & 6.2) along the axis of the main pancreatic duct, and the weight of each portion recorded. One half of each gland was then digested by the manual method, and the other half by the automated procedure. Digestions were performed simultaneously by two teams of operators.

**Manual Digestion**

A similar method to that used in chapters four and five was used. A biopsy was taken from the region of the pancreas that was seen to distend first, usually the tail, and divided into five pieces. Each piece was incubated at 35°C in a universal container with 1ml pre-warmed dithizone (Sigma; 1.2 mmol/l in 5 per cent (v/v) dimethyl sulphoxide – MEM and 1 per cent bovine serum albumin (BSA)). The pancreas was similarly incubated in MEM at 35°C. After five minutes incubation, a universal container was removed from the water bath, shaken for thirty seconds and the contents examined by incident white-light microscopy. If a single cleaved (i.e. totally free of exocrine tissue) islet was seen, the pancreas was removed from incubation. If no cleaved islets were present, further biopsies were examined at intervals of 2.5 – 7.5 minutes.

When cleaved islets were seen, the pancreas was removed from the incubation medium and placed into one half of a kidney bowl divided by a 1-mm mesh, and gently teased apart with forceps. The digest that passed through the mesh was aspirated and passed through a 500-µm mesh into MEM at 4°C containing twenty per cent newborn calf serum (NBCS; Advanced Protein Products, Brierley Hill, UK) and 0.004 mg/ml DNAase. A further 50 ml MEM at 35°C containing ten per cent NBCS was added to the kidney bowl, and the teasing and aspiration continued for ten to thirty minutes until digestion ceased.
Automated Digestion

The automated method utilized digestion of the collagenase-distended pancreas within an enclosed stainless steel digestion chamber (Fig 6.3). The half-pancreas was loaded into the chamber, together with seven stainless steel ball-bearings, of 1-cm diameter. The chamber was divided in two by a stainless steel mesh (Fig 6.4) with 355-μm pores. A circuit was set up as shown in the diagram below using polyvinyl chloride tubing (COBE Laboratories, Gloucester, UK), Fig 6.5. MEM was recirculated at a rate of 50 ml/min through the chamber with a heating circuit, (Fig 6.6) maintaining the temperature in the chamber at 35°C during this first (recirculation) phase.

![Diagram of the digestion process](image)

Once the medium started to recirculate, the chamber was agitated by an attached mechanical shaker, Fig 6.7, producing 300 vertical oscillations per minute. The temperature in the chamber was maintained at 35°C. At two to five minute intervals, 5ml samples of the medium emerging from the chamber were taken from a side-port of the tubing, stained with dithizone, and examined for the presence of cleaved islets. When approximately ten islets were seen in a single microscopic field of x100 magnification, (Fig 6.8), the circuit was opened, enabling fresh medium (MEM, 10 per cent DNAase) at 4°C to enter the chamber at a rate of 300ml/min. This was the second (collection) phase. During this phase the chamber effluent was collected at 4°C into
MEM containing twenty per cent NBCS. Digestion was judged complete when no further islets were seen in the biopsies from the chamber effluent.

**Islet Quantification**

The non-distended weight of the two portions of the pancreas after longitudinal sectioning was calculated from the overall ratio of non-distended : distended weight, multiplied by the weight of the distended portion of the gland. Islet yields are expressed per gram non-distended pancreas.

The digest produced by each method was washed three times in MEM with ten per cent NBCS, and suspended in a total volume of 50ml. Five 200-µl samples were taken from the 50-ml tube, maintaining the tissue in suspension between samples by continuous shaking. Each of these samples was diluted tenfold with MEM in five separate universal containers, and two 100-µl samples taken from these and separately placed on to a Petri dish, to give a total of ten samples from each digestion. Dithizone (100µl, diluted to one tenth normal concentration) was added, the number of islets >50µm diameter counted in each sample, (Figs 6.9 & 6.10), and the total number of islets calculated from the mean number per sample multiplied by the dilution factor.

The mean volume of an islet was estimated by measuring 100 consecutive islet diameters using an eyepiece micrometer, and calculating the volume of each islet (0.524 x diameter³, assuming that islets are spherical). The total volume of islet tissue isolated was calculated as the product of the mean volume of an islet and their total number. Islet yield was corrected to '105-µm islet equivalents' by dividing the total islet tissue volume by the volume of an islet of 105µm in diameter (6.1 x 10⁻⁴ mm³). This latter correction was made because studies from this laboratory on the size distribution of islets within pancreata from donor pigs, have shown that 105-µm is the mean diameter of islets in histological section. This correction, therefore, equates to the 150-µm islet equivalent correction reported for human islet isolation studies. The percentage of cleaved (completely free of any surrounding exocrine tissue) islets in the digest was estimated from inspection of the final samples.

**Islet Purification**

After removal of the above samples, the final digests were centrifuged at 100g for two minutes and 100-ml aliquots suspended in 1 ml BSA (density 1.095 g/cm³) in conical test tubes. These were overlain with a continuous linear density gradient of BSA, generated by a gradient maker (Hoefer Scientific Instruments, San Francisco,
California, USA), containing in its chambers BSA of densities 1.069 and 1.095 g/cm³, osmolality 400mOsmol/kg.

These gradients were then centrifuged at 500g for 20 min at 22°C and eleven x 1-ml aliquots aspirated carefully, from the top to bottom of the gradients. After washing these aliquots in MEM and resuspending the tissue in 2-ml MEM, each sample was sonicated for 20s, 1-ml being stored for amylase assay while the other 1-ml underwent acid-alcohol extraction and subsequent storage for insulin assay. Amylase assay was by Phadebas Amylase Test Kit (Pharmacia Diagnostics, Uppsala, Sweden), and insulin was assayed by radioimmunoassay.

The efficiency of density gradient purification was assessed by plotting the cumulative percentage of amylase and insulin in these aliquots against density. The degree of exocrine (amylase) contamination at an islet (insulin) yield of 70 per cent was used as the criterion for comparison because this gave an estimate of islet purity when a reasonable yield of islets was obtained.

The percentage of insulin in the ‘pellet’ i.e. the lower 3 aliquots of the gradient, was also compared between digestion methods as an index of islet / exocrine separation.

Statistical Analysis
Values are given as median (range); comparisons were performed using the Wilcoxon signed rank test for paired data.
6.3 Results

During seven consecutive “dual” digestions, 63g (50g-75g) of porcine pancreas was split between the two digestion methods in each preparation, giving a digest volume of 4ml (1-9ml) after standard digestion and 5.5ml (3-9ml) after automated digestion.

**Islet Quantification**

Islet quantification was performed as previously described, and is summarized in Table 6.1:

Full details of this data are given in Tables 6.2 and 6.3 and comparisons are shown graphically in Figures 6.11–6.17

All results are median (range).

<table>
<thead>
<tr>
<th>Table 6.1</th>
<th>Standard Method</th>
<th>Automated Method</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of islets per gram pancreas</td>
<td>2698 (2013-5591)</td>
<td>4014 (2733-6920)</td>
<td>0.076</td>
</tr>
<tr>
<td>Mean Volume of an islet (x10^-4mm^3)</td>
<td>3.64 (2.12-6.34)</td>
<td>9.36 (4.39-11.20)</td>
<td>0.022</td>
</tr>
<tr>
<td>Total Islet Tissue Volume (mm^3 per gram pancreas)</td>
<td>1.07 (0.46-1.92)</td>
<td>3.56 (1.39-5.30)</td>
<td>0.022</td>
</tr>
<tr>
<td>105-μm Islet Equivalents per gram pancreas</td>
<td>1766 (759-3168)</td>
<td>5875 (2294-8746)</td>
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<tr>
<td>Cleavage Index (%)</td>
<td>82 (78-92)</td>
<td>92 (89-99)</td>
<td>0.035</td>
</tr>
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</table>

Values are median (range). *Wilcoxon signed rank test for paired data.

The absolute yield of islets per gram pancreas digested did not differ significantly between the two methods. However, there were marked differences between the two procedures with respect to the other parameters of digestion studied. The islets produced by the automated method were significantly larger than those from the manual technique, as reflected by the higher mean volume of an islet, and so the former method produced a larger total volume of islet tissue per gram pancreas digested. This is most clearly expressed as the number of 105-μm islet equivalents per gram, which was significantly greater for the automated technique. Finally, the cleavage index was significantly higher with the automated technique.
Islet Purification

Samples of each digest were placed on 400mOsmol/kg BSA continuous density gradients as described. After centrifugation, aspiration and assay, graphs were constructed as in chapters 5 & 6. These graphs are given in Figures 6.20-6.33.

These results are summarized below in Table 6.4. The endocrine / exocrine separation results for each preparation are shown directly compared in Figs 6.34 and 6.35

Exocrine Contamination (%) at 70% Insulin

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</table>

These results of the BSA density gradient purification studies showed that the median (range) amylase concentration at a 70 per cent insulin yield for the automated method was 50 (4-95) per cent and for the manual method 34 (9-90) per cent. $P = 1.0$.

No statistical difference was shown using the Wilcoxon signed rank test for paired data.

Insulin (%) in Pellet

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</table>

No statistical difference was shown using the Wilcoxon signed rank test for paired data on non-parametric testing of the data.
Porcine islets (Ricordi 1986, Ricordi 1990) are very fragile; the pig pancreas is therefore a good model in which to test the efficacy of methods purporting to produce effective digestion. The porcine pancreata used in the present study were obtained from abattoir pork pigs under normal conditions of abattoir operation. Although porcine islet isolation can be optimized by using a particular breed and age of pig and minimizing warm ischaemia time (Calafiore 1990, Ricordi 1990(c)), the abattoir pig remains a readily available source of material with which to conduct studies applicable to human islet transplantation.

A successful method for pancreatic digestion must disrupt the gland whilst preserving the structure of the islets themselves. The intraductal route of collagenase delivery ensures that the enzyme is delivered directly into the pancreatic acini, thus acting first to dissociate exocrine tissue from endocrine tissue. The key to production of large numbers of intact islets lies in the exposure of the islets to collagenase for a critical time, long enough to allow their release from surrounding exocrine tissue, but short enough to prevent digestion of the islets themselves. Because different pancreata vary widely with respect to their optimal collagenase time, and because islets are susceptible to mechanical trauma, many existing methods of digestion are inadequate and produce marked islet fragmentation. This is particularly true in the case of porcine islet isolation.

The two methods compared in this study are recent adaptations of collagenase digestion, designed to attempt to overcome these problems. The manual, biopsy-based method has the advantage of simplicity and requires little specialized equipment. It is the only non-automated technique based on individual ‘tailoring’ of the exposure of each pancreas to collagenase.

The automated method has the advantage of the continual circulation of medium, allowing the progressive liberation of islets and their immediate protection from collagenase once liberated. It is however, relatively complex to set up.

In this prospective study a comparison of these two widely used techniques was undertaken, dividing each pancreas and digesting the two halves by each method simultaneously. The size and distribution of islets is known to vary from the head to the tail of the gland (Marchetti 1990) and the pancreata were therefore divided longitudinally to produce a similar islet population between the two methods. Thus,
each pancreas acted as its own control, minimizing any adverse effects arising from the natural variation in collagenase digestion between different pancreata.

The absolute numbers of islets >50\textmu m in diameter produced by the two techniques did not differ significantly and are comparable to those achieved by other groups using these methods (Ricordi 1990 (b), Ricordi 1990 (c), Calafiore 1990). Care must be taken when making such comparisons because porcine islets vary greatly with the age and strain of pig used (Socci 1989), particularly with respect to size and fragility. It is not possible to measure islet fragmentation directly because small fragments are lost to waste during the washing stages of the isolation process. However it can be concluded, because the mean volume of an islet, the total islet tissue volume and the number of 105-\textmu m islet equivalents were significantly higher with the automated method, that most islets from the manual digestion were small and fragmented. In comparison, islets isolated by the automated method remained intact; their mean volume was comparable to the mean histological islet volume in the pancreas of donor pigs (6.1 x 10^{-4} \text{ mm}^3).

The islet cleavage index was significantly higher with the automated method. This finding has important implications for subsequent islet purification because the density of uncleaved islets approaches that of adherent exocrine tissue, making it impossible to purify them by isopycnic density-gradient centrifugation. This result, taken in conjunction with the findings that the median amylase concentration at a 70 per cent insulin yield was no different between the automated and manual methods, and that there was a greatly increased volume of islet tissue in the digest from the automated procedure, means that the islet yield per gram original pancreas will be greater following purification with the automated than the manual method.

In conclusion, this prospective study has shown that, compared with a standard manual method, the automated technique more than trebled the volume of islet tissue isolated from the porcine pancreas. Although the automated digestion method is relatively complex to set up, the greatly improved yield of intact islets justifies its routine use for porcine islet isolation.
### Manual Digestion

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<th>mm³</th>
<th>x10⁻⁴ mm³</th>
<th>Digest Volume per gram</th>
<th>Islets Cleavage Index</th>
<th>Mean Islet Diameter</th>
<th>Mean Islet Tissue per gram</th>
<th>Mean Islet Volume</th>
<th>Islet Equivs per gram</th>
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### Automated Digestion

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<th>Digest Volume per gram</th>
<th>Islets Cleavage Index</th>
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Fig 6.1  The Porcine Pancreas, Distended with Collagenase, is Divided Along the Axis of the Main Pancreatic Duct.

Fig 6.2  The Two Halves of the Pancreas, destined for Manual and Automated Digestion respectively.
Fig 6.3 The Lower Component of the Stainless Steel Digestion Chamber Containing One Half-Pancreas

Fig 6.4 The Stainless Steel Mesh, Pore Size 300μm, Separating the Upper and Lower Components of the Digestion Chamber.
Fig 6.5  The Automated Digestion Circuit as set up in the Laboratory.

Fig 6.6  Close-Up View of the Heating Circuit.
A Standard Blood Heat-Exchanger is connected to a Water Bath. MEM Flowing through the Circuit is either Warmed or Diverted.
Fig 6.7  The Digestion Chamber connected to the Mechanical Shaking Device. A Temperature Sensor at the Outlet of the Chamber connects to a Temperature Meter on top of the Shaking Device Control Panel.
Fig 6.8  Tissue Sample x50 from the Biopsy Port during the Recirculation Phase (Phase 1) of Automated Digestion. Cleaved Islets are present. Dithizone Stained.

Fig 6.9  Digest x50 Produced by the Manual Digestion Method.
Fig 6.10  Digest x50 Produced by the Automated Digestion Method.
Figure 6.11

**FINAL DIGEST VOLUME**

![Bar chart showing final digest volume for different preparations.](#)

**PREPARATION No.**

Fig 6.12

**No. ISLETS PER GRAM PANCREAS**

![Bar chart showing islets per gram for different preparations.](#)

**PREPARATION No.**
Fig 6.13

**MEAN ISLET DIAMETER**

<table>
<thead>
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<th>STD DIGESTION</th>
<th>AUTO DIGESTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 2 3 4 5 6 7</td>
<td></td>
</tr>
</tbody>
</table>

Fig 6.14

**MEAN ISLET VOLUMES**

From mean of 100 measured volumes per digestion

<table>
<thead>
<tr>
<th>STD DIGESTION</th>
<th>AUTO DIGESTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 2 3 4 5 6 7</td>
<td></td>
</tr>
</tbody>
</table>
Fig 6.15

ISLET EQUIVALENTS PER GRAM PANCREAS

Calculated using the volume of a 105micron diameter islet

STD DIGESTION
AUTO DIGESTION

Equivs per gram

Fig 6.16

TOTAL ISLET TISSUE PER GRAM PANCREAS

STD DIGESTION
AUTO DIGESTION

mm3 / gram
Fig 6.17

% FULLY CLEAVED ISLETS IN DIGEST

STD DIGESTION
AUTO DIGESTION

PREPARATION No.
**Fig 6.18**

**PREP 1. STANDARD DIGESTION**

400mOsmol BSA Gradient Separation

- % Amylase at 70% Insulin: 34%
- % Insulin in Pellet: 32%

**Fig 6.19**

**PREP 1. AUTOMATED DIGESTION**

400mOsmol BSA Gradient Separation

- % Insulin at 70% Insulin: 25%
- % Insulin in Pellet: 39%
Fig 6.20

**PREP 2. STANDARD DIGESTION**

400mOsmol BSA Gradient Separation

% Amylase at 70% Insulin: 52%
% Insulin in Pellet: 28%

Fig 6.21

**PREP 2. AUTOMATED DIGESTION**

400mOsmol BSA Gradient Separation

% Amylase at 70% Insulin: 50%
% Insulin in Pellet: 25%
Fig 6.22

**PREP 3. STANDARD DIGESTION**

400mOsmol BSA Gradient Separation

- % Amylase at 70% Insulin: 9%
- % Insulin in Pellet: 21%

Cumulative Insulin
Cumulative Amylase
Absolute Insulin

Fig 6.23

**PREP 3. AUTOMATED DIGESTION**

400mOsmol BSA Gradient Separation

- % Amylase at 70% Insulin: 4%
- % Insulin in Pellet: 13%

Cumulative Insulin
Cumulative Amylase
Absolute Insulin
**PREP 4. STANDARD DIGESTION**

400mOsmol BSA Gradient Separation

- % Amylase at 70% Insulin: 56%
- % Insulin in Pellet: 19%

**PREP 4. AUTOMATED DIGESTION**

400mOsmol BSA Gradient Separation

- % Amylase at 70% Insulin: 36%
- % Insulin in Pellet: 5%
**Fig 6.26**

**PREP 5. STANDARD DIGESTION**

400mOsmol BSA Gradient Separation

% Amylase at 70% Insulin: 90%
% Insulin in Pellet: 60%

- Cumulative Insulin
- Cumulative Amylase
- Absolute Insulin

**Fig 6.27**

**PREP 5. AUTOMATED DIGESTION**

400mOsmol BSA Gradient Separation

% Amylase at 70% Insulin: 95%
% Insulin in Pellet: 69%

- Cumulative Insulin
- Cumulative Amylase
- Absolute Insulin
Fig 6.28

**PREP 6. STANDARD DIGESTION**

400mOsmol BSA Gradient Separation

- % Amylase at 70% Insulin: 18.5%
- % Insulin in Pellet: 39%

Cumulative Insulin
Cumulative Amylase
Absolute Insulin

Fig 6.29

**PREP 6. AUTOMATED DIGESTION**

400mOsmol BSA Gradient Separation

- % Amylase at 70% Insulin: 61%
- % Insulin in Pellet: 50%

Cumulative Insulin
Cumulative Amylase
Absolute Insulin
Fig 6.30

PREP 7. STANDARD DIGESTION

400mOsmol BSA Gradient Separation

% Amylase at 70% Insulin: 27%
% Insulin in Pellet: 19%

Fig 6.31

PREP 7. AUTOMATED DIGESTION

400mOsmol BSA Gradient Separation

% Amylase at 70% Insulin: 62%
% Insulin in Pellet: 20%
Fig 6.32

% AMYLASE AT 70% INSULIN
Median values after 7 consecutive preparations

Fig 6.33

% INSULIN IN PELLET
Median values after 7 consecutive preparations
CHAPTER 7

The Optimization Of The Automated Digestion Method

7.1 Introduction
7.2 Methods
7.3 Results
7.4 Discussion
7.5 Figures and Tables
The results of the work presented in the previous chapter show that automated digestion is a more effective method of porcine pancreatic digestion than the manual, biopsy-based technique. It is probably the most effective method available at present.

The production of large amounts of islet tissue in the digest is certainly greatly enhanced by the automated method as it stands at present. The avoidance of over-digestion results in a larger mean islet volume, which is reflected in the increased total volume of islet tissue in the digest. The only disappointing facet to automated digestion is that islet separation and purity of the final islet preparation does not seem to be improved. Although total islet yield from any pancreas digested using the automated method would be increased compared to manual digestion, the degree of exocrine contamination would make this tissue unsuitable for transplantation (Gray 1986).

Examination and assay of the continuous density gradients described in the last chapter, showed no difference in final purity between the digestion methods tested. Although avoidance of over-digestion prevents much islet break-up, significant fragmentation is still occurring, as demonstrated by the many tiny islet fragments visible at all levels in the gradients, after both manual and automated digestion. If it is postulated that islet integrity is mandatory for determining a significant density difference between islets and exocrine, then preservation of islet integrity is essential for successful density gradient separation. On a purely anecdotal basis it was noticed that the preparations with the least islet fragmentation in the digest were those which underwent the most successful islet purification.

Porcine islets are significantly more fragile than human or most other animal islets (Ricordi, 1986). With loose vascular channels running through these irregular islets, they are very vulnerable to both enzymatic and mechanical trauma. The automated digestion method does remove islets from over-exposure to collagenase, but the vigorous shaking of the digestion chamber and the action of the ball-bearings seems likely to result in significant damage to the islets lying free in the chamber. Scrupulous attention to the temperature within the chamber is also necessary, as temperatures of 40°C or above in the in vitro situation have always resulted in islet fragmentation.

These pilot studies were designed to avoid unnecessary mechanical trauma to the islets during automated digestion of the porcine pancreas. They were performed with
alterations to the concentration of the collagenase, and the timing, duration and force of the shaking of the chamber.

7.2 Methods

Pancreatic Tissue
Porcine pancreata were obtained as described in Chapter Six.

Collagenase
Serva collagenase was made up in HBSS and DNAase as described in Chapter Six. For each automated digestion, 200ml of collagenase was prepared at concentrations of 2mg/ml, 4mg/ml and 8mg/ml. Collagenase was then stored at -20°C until needed. Pancreata were distended with collagenase as previously described and any excess collagenase, usually 50-80ml, was poured into the digestion chamber.

Digestion Techniques
Porcine pancreata were digested by the following automated digestion methods:

Automated Method 1 (AM1)
Three pancreata underwent automated digestion using collagenase at a concentration of 2mg/ml without any shaking of the chamber in either phase one (recirculation) or phase two (collection).

Automated Method 2 (AM2)
Three pancreata underwent automated digestion using collagenase at a concentration of 4mg/ml with intermittent shaking in phase one and continuous shaking in phase two.

Automated Method 3 (AM3)
Three pancreata underwent automated digestion using collagenase at a concentration of 4mg/ml with only intermittent shaking, in both phases one and two.
Automated Method 4 (AM4)

3 pancreata underwent automated digestion using collagenase at a concentration of 8mg/ml and only intermittent shaking, in both phases one and two.

Intermittent shaking in phase one entailed a two second shake every two minutes after ten minutes of ‘static’ recirculation (no shaking at all). Each ‘shake’ was immediately followed by the aspiration of a 5ml sample for microscopic examination with dithizone, to look for the presence of cleaved islets.

Intermittent shaking in phase two entailed a 5 second shake every two minutes, until digest ceased to flow out of the digestion chambers.

Islets in the digest produced in each preparation were quantified as described in Chapter Six. Samples of each digest produced were subjected to density gradient centrifugation using BSA at 400-mOsmol/kg as described in Chapter Six. Insulin and amylase extractions were again performed on the aliquots of the gradients as described, and separation graphs drawn as previously presented.
7.3 Results

Islet Quantification

The results are summarized in Table 7.1, giving the mean values of the three preparations performed using each method. Full details of the data are given in Tables 7.2 - 7.5 and are expressed graphically in Figs 7.1 - 7.5.

<table>
<thead>
<tr>
<th>Auto Method</th>
<th>Mass (g)</th>
<th>Digest Volume (ml)</th>
<th>Islets per gram</th>
<th>Mean Diameter (mm)</th>
<th>Mean Islet Volume (x10^-4 mm^3) per gram</th>
<th>Islet Tissue per gram</th>
<th>Islet Equivs per gram</th>
</tr>
</thead>
<tbody>
<tr>
<td>AM1</td>
<td>60</td>
<td>0.66</td>
<td>775</td>
<td>89</td>
<td>4.05</td>
<td>0.32</td>
<td>522</td>
</tr>
<tr>
<td>AM2</td>
<td>82</td>
<td>11.8</td>
<td>8980</td>
<td>101</td>
<td>7.9</td>
<td>4.54</td>
<td>7497</td>
</tr>
<tr>
<td>AM3</td>
<td>63</td>
<td>26.1</td>
<td>7566</td>
<td>116</td>
<td>16.2</td>
<td>12.2</td>
<td>20196</td>
</tr>
<tr>
<td>AM4</td>
<td>89</td>
<td>12.3</td>
<td>5775</td>
<td>74</td>
<td>2.97</td>
<td>1.69</td>
<td>2300</td>
</tr>
</tbody>
</table>

Automated digestion method three (AM3), using 4mg/ml collagenase and an automated digestion with minimal mechanical trauma gave significantly greater yield of islet tissue in the digest, reflected in the greater mean islet volume, total islet tissue per gram and number of islet equivalents per gram. Significance of p < 0.01 was reached using the paired Students t-test against all the other automated methods for these criteria.

Islet Purification

Samples of each digest from each automated digestion method were subjected to 400-mOsmol/kg BSA density gradient separation as described in Chapter Six. Aliquots of the BSA gradients were assayed and separation graphs drawn as previously described.

The digest islet quantification results for AM1 were very poor, representing such complete lack of digestion, that no attempts at islet purification were made. No data are presented.

The endocrine/exocrine separation graphs for AM2, AM3 and AM4 are given in Figs 7.6 - 7.14. The mean values of the data are given below in Table 8.2 and the data is given fully in Tables 7.10 - 7.12, with graphs in Figs 7.15 & 7.16.
Table 8.2

<table>
<thead>
<tr>
<th>Automated Method</th>
<th>% Amylase at 70% In</th>
<th>% Insulin in Pellet</th>
</tr>
</thead>
<tbody>
<tr>
<td>AM2</td>
<td>30%</td>
<td>21%</td>
</tr>
<tr>
<td>AM3</td>
<td>63%</td>
<td>93%</td>
</tr>
<tr>
<td>AM4</td>
<td>61%</td>
<td>26%</td>
</tr>
</tbody>
</table>

A dramatic effect on endocrine separation is seen with digest prepared using AM3. Paired t-testing shows a significant difference (p < 0.01) between method 3 and both methods 2 and 4, for both criteria.

7.4 Discussion

Porcine islets are amongst the most difficult to isolate intact as they are much more fragile than other islets (Ricordi et al 1986), possibly because of their irregular structure and the presence of what seem like vascular channels within the islets. Recent studies into the extracellular matrix of human, dog, rodent and porcine pancreata (Van Deijnen et al 1991) showed the presence of definite collagen capsules around human, canine and rodent islets, but the total absence of any capsule around porcine islets. As a rule the porcine islet periphery is in direct contact with the exocrine tissue, making it very susceptible to collagenase damage as the acinar structure is broken up during intraductal enzyme administration. The lack of capsule also contributes towards susceptibility to mechanical trauma.

Over the years a number of techniques for dispersing the pancreas have been tried, varying from hand-dissection of islets to ultrasonic destruction. It has been clear that the most effective methods have involved the action of collagenase, administered intraductally (Gray et al 1984), and the mechanical dissociation of the tissue. Many methods have used both these techniques either alone or in combination, but tests on the effective contribution of each component on efficient dispersal have seldom been documented. Excessive enzymatic action on the islets themselves will damage them, as will excessive mechanical trauma.

If islet integrity is essential for maintenance of essential physical properties, it can hardly be surprising that effective density gradient separation of islets from exocrine depends so much on the islets in the digest being undamaged. Certainly the evidence from the experiments presented in this thesis indicates that with avoidance of
overdigestion and elimination of unnecessary mechanical trauma, numerous large intact islets can be found in the digest, (Figs 7.17–7.19).

These experiments were designed to give a general indication of the minimum enzymatic and mechanical trauma necessary for the production of a good quality pancreatic digest. Combinations of collagenase concentration and degree of mechanical agitation were studied. For purely financial reasons it was not possible to study every feasible combination of these variables and it is hoped that in the future these studies may be supported by further work.

Automated digestion without mechanical agitation, using a low collagenase concentration was inadequate, as demonstrated by the very poor yield of islet tissue when the pancreata were digested using AM1. A few tiny islets, mean diameter 89µm, were released, but little else. Two empirical preparations using a higher concentration of collagenase, 4mg/ml, again without agitation, similarly did not produce any effective digestion, with the intact pancreas remaining virtually intact in the chamber at the end of the process. No data are presented.

The automated digestion method of Chapter Six involved continuous agitation of the chamber (containing steel bearings and the pancreas) during both the re-circulation phase (phase one) and the dilution phase (phase two). The digest produced still contained many fragments of broken-up islets as witnessed by the data in the previous chapter, and attempted separation was no better than with islets isolated by an imperfect manual method. The next method to be formally tested, AM2, therefore attempted the automated digestion of a pancreas distended with collagenase at 4mg/ml, using intermittent agitation in recirculation and continuous agitation during dilution. Results were similar to those produced in chapter seven, with production of 4.54mm³ islet tissue per gram pancreas, mean islet diameter of 101µm, and a mean islet volume of 7.9 x10^-4mm³.

With the collagenase concentration of 4mg/ml producing effective albeit excessive digestion in the previous studies, AM3 was designed to minimize mechanical trauma in the presence of an effective enzyme concentration. Intermittent agitation in both phases, combined with the effective enzyme concentration of 4mg/ml produced dramatically improved results. Digest volume was increased to a mean of 26ml, the tubing of the circuit actually becoming opaque as the tissue poured out of the chamber. A mean value of 12.2mm³ islet tissue per gram pancreas was produced in the digest, the mean islet diameter being 116µm and the mean islet volume 16.2 x10^-4mm³. These
figures exceed the mean histological measurements but histologically, the majority of porcine islets are <50μm in diameter, contributing significantly to, lower mean islet diameters. No islets produced by digestion were ‘larger’ than those actually existing, even in low numbers, in the histological sections from identical pigs. It appears likely that many of the ‘intact’ islets measured in previous studies are fragments of larger islets, the smaller islets being completely dispersed and destroyed by the enzymatic and mechanical processes.

Automated method four (AM4), using a higher concentration of collagenase in conjunction with intermittent agitation in both phases, overdigested the pancreata. Mean values of 1.69mm³ per gram islet tissue, with a mean islet diameter of 74μm and mean islet volume of 2.97 x10^{-4}mm³ differed very significantly from those produced by AM3.

The most promising results however were those of the purification of islets produced by the four methods. The large, ‘intact’ islets produced by AM3 were far more capable of purification, separating extremely well on a 400mOsm /kg BSA density gradient (Figs 7.20 & 7.21), with a mean contaminating amylase level of 6.3% if 70% of the islets were isolated. Only 9% of the total islet tissue did not separate from the exocrine. These values differed significantly (p<0.01) from those of islets separated in the digest produced by the other methods.

Statistical analysis has been applied to the data presented in this chapter but it is realised that the term “significance” does not really apply to studies with such small numbers as these. There was little doubt that method AM3 was a vast improvement on the previous technique but financial considerations (the expense of collagenase) have made the statistical proof unavailable.

These results do bear out the theory that endocrine can only be expected to separate well from the exocrine if the islets are intact. The combination of an effective collagenase concentration, in combination with effective but minimal mechanical dispersal, is essential for effective pancreatic digestion, in the porcine pancreas.
## 7.5 Figures and Tables

### Table 7.2

**AUTOMATED METHOD 1 (AMI)**

<table>
<thead>
<tr>
<th>Prep No.</th>
<th>Mass (g)</th>
<th>Digest Volume (ml)</th>
<th>Islets per gram</th>
<th>Mean Diameter (μm)</th>
<th>Mean Islet Volume ($\times 10^{-4} mm^3$)</th>
<th>Islet Tissue (mm$^2$)</th>
<th>Islet Equivs per gram</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>47</td>
<td>0.5</td>
<td>765</td>
<td>86</td>
<td>3.22</td>
<td>0.25</td>
<td>412</td>
</tr>
<tr>
<td>2</td>
<td>58</td>
<td>1.0</td>
<td>972</td>
<td>97</td>
<td>4.88</td>
<td>0.41</td>
<td>677</td>
</tr>
<tr>
<td>3</td>
<td>63</td>
<td>0.5</td>
<td>588</td>
<td>84</td>
<td>4.86</td>
<td>0.29</td>
<td>476</td>
</tr>
</tbody>
</table>

### Table 7.3

**AUTOMATED METHOD 2 (AM2)**

<table>
<thead>
<tr>
<th>Prep No.</th>
<th>Mass (g)</th>
<th>Digest Volume (ml)</th>
<th>Islets per gram</th>
<th>Mean Diameter (μm)</th>
<th>Mean Islet Volume ($\times 10^{-4} mm^3$)</th>
<th>Islet Tissue (mm$^2$)</th>
<th>Islet Equivs per gram</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>64</td>
<td>10.0</td>
<td>8049</td>
<td>94</td>
<td>11.3</td>
<td>5.71</td>
<td>9422</td>
</tr>
<tr>
<td>2</td>
<td>57</td>
<td>12.0</td>
<td>6038</td>
<td>103</td>
<td>6.23</td>
<td>3.77</td>
<td>6221</td>
</tr>
<tr>
<td>3</td>
<td>61</td>
<td>13.5</td>
<td>6854</td>
<td>106</td>
<td>6.96</td>
<td>4.15</td>
<td>6848</td>
</tr>
</tbody>
</table>
### Table 7.4

**AUTOMATED METHOD 3 (AM3)**

<table>
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<tr>
<th>Prep No.</th>
<th>Mass (g)</th>
<th>Digest Volume (ml)</th>
<th>Islets per gram</th>
<th>Mean Diameter (μm)</th>
<th>Mean Islet Volume (x10^-4 mm^3)</th>
<th>Tissue Islet Equivs per gram</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>63</td>
<td>25.0</td>
<td>7887</td>
<td>116</td>
<td>16.4</td>
<td>12.9</td>
</tr>
<tr>
<td>2</td>
<td>63</td>
<td>39.0</td>
<td>8174</td>
<td>120</td>
<td>17.4</td>
<td>14.2</td>
</tr>
<tr>
<td>3</td>
<td>57</td>
<td>15.4</td>
<td>6666</td>
<td>111</td>
<td>14.4</td>
<td>9.6</td>
</tr>
</tbody>
</table>

### Table 7.5

**AUTOMATED METHOD 4 (AM4)**

<table>
<thead>
<tr>
<th>Prep No.</th>
<th>Mass (g)</th>
<th>Digest Volume (ml)</th>
<th>Islets per gram</th>
<th>Mean Diameter (μm)</th>
<th>Mean Islet Volume (x10^-4 mm^3)</th>
<th>Tissue Islet Equivs per gram</th>
</tr>
</thead>
<tbody>
<tr>
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<td>63</td>
<td>12.0</td>
<td>5669</td>
<td>79</td>
<td>3.42</td>
<td>1.93</td>
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<tr>
<td>2</td>
<td>67</td>
<td>11.4</td>
<td>4708</td>
<td>73</td>
<td>2.99</td>
<td>1.41</td>
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<tr>
<td>3</td>
<td>60</td>
<td>14.0</td>
<td>6986</td>
<td>69</td>
<td>2.51</td>
<td>1.75</td>
</tr>
</tbody>
</table>
Fig 7.1

**FINAL VOLUME OF DIGEST PRODUCED**

Mean of 3 Preparations per digestion method

![Bar chart for Digest Volume (ml)]

<table>
<thead>
<tr>
<th>Digest Volume (ml)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5</td>
<td>10</td>
<td>25</td>
<td>20</td>
</tr>
</tbody>
</table>

AUTOMATED DIGESTION METHOD

Fig 7.2

**NO. ISLETS PER GRAM PANCREAS IN DIGEST**

Mean of 3 Preparations per digestion method

![Bar chart for Islets per gram]

<table>
<thead>
<tr>
<th>Islets per gram</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>15</td>
<td>40</td>
<td>80</td>
<td>60</td>
</tr>
</tbody>
</table>

AUTOMATED DIGESTION METHOD
Fig 7.3

**FINAL VOLUME OF DIGEST PRODUCED**

*Mean of 3 Preparations per digestion method*

![Bar graph showing final volume of digest produced for different digestion methods.](image)

**Fig 7.4**

**ISLET TISSUE PER GRAM PANCREAS IN DIGEST**

*Mean of 3 Preparations per digestion method*

![Bar graph showing islet tissue per gram for different digestion methods.](image)
Fig 7.5

ISLET EQUIVALENTS PER GRAM PANCREAS IN DIGEST

Mean of 3 Preparations per digestion method.
105 micron diameter equivalents.

AUTOMATED DIGESTION METHOD
Fig 7.6

AUTO METHOD 2. PREPARATION 1

400mOsmol BSA Gradient Separation

Fig 7.7

AUTO METHOD 2. PREPARATION 2

400mOsmol BSA Gradient Separation
Fig 7.8

AUTO METHOD 2. PREPARATION 3

400mOsmol BSA Gradient Separation

Cumulative Insulin
Cumulative Amylase
Absolute Insulin
Fig 7.9

**AUTO METHOD 3 PREPARATION 1**

*400mOsmol BSA Gradient Separation*

![Graph showing Cumulative Insulin, Cumulative Amylase, and Absolute Insulin](image1)

Fig 7.10

**AUTO METHOD 3. PREPARATION 2**

*400mOsmol BSA Gradient Separation*

![Graph showing Cumulative Insulin, Cumulative Amylase, and Absolute Insulin](image2)
Fig 7.11

AUTO METHOD 3. PREPARATION 3

400mOsmol BSA Gradient Separation

Cumulative Insulin
Cumulative Amylase
Absolute Insulin
Fig 7.12

AUTO METHOD 4. PREPARATION 1

400mOsmol BSA Gradient Separation

BSA Density (g/cm³)

0 %

100 %

Cumulative Insulin
Cumulative Amylase
Absolute Insulin

Fig 7.13

AUTO METHOD 4. PREPARATION 2

400mOsmol BSA Gradient Separation

BSA Density (g/cm³)
Fig 7.14

AUTO METHOD 4. PREPARATION 3

400mOsmol BSA Gradient Separation

- Cumulative Insulin
- Cumulative Amylase
- Absolute Insulin

BSA Density (g/cm³)
### Table 7.10
**AUTOMATED DIGESTION METHOD 2 (AM2)**

<table>
<thead>
<tr>
<th>Prep No.</th>
<th>% Amylase at 70%</th>
<th>% Insulin in Pellet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amylase at 70%</td>
<td>Insulin</td>
</tr>
<tr>
<td>1</td>
<td>39%</td>
<td>21%</td>
</tr>
<tr>
<td>2</td>
<td>19%</td>
<td>12%</td>
</tr>
<tr>
<td>3</td>
<td>32%</td>
<td>30%</td>
</tr>
</tbody>
</table>

### Table 7.11
**AUTOMATED DIGESTION METHOD 3 (AM3)**

<table>
<thead>
<tr>
<th>Prep No.</th>
<th>% Amylase at 70%</th>
<th>% Insulin in Pellet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amylase at 70%</td>
<td>Insulin</td>
</tr>
<tr>
<td>1</td>
<td>8%</td>
<td>12%</td>
</tr>
<tr>
<td>2</td>
<td>6%</td>
<td>10%</td>
</tr>
<tr>
<td>3</td>
<td>5%</td>
<td>6%</td>
</tr>
</tbody>
</table>

### Table 7.12
**AUTOMATED DIGESTION METHOD 4 (AM4)**

<table>
<thead>
<tr>
<th>Prep No.</th>
<th>% Amylase at 70%</th>
<th>% Insulin in Pellet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amylase at 70%</td>
<td>Insulin</td>
</tr>
<tr>
<td>1</td>
<td>66%</td>
<td>31%</td>
</tr>
<tr>
<td>2</td>
<td>59%</td>
<td>34%</td>
</tr>
<tr>
<td>3</td>
<td>58%</td>
<td>26%</td>
</tr>
</tbody>
</table>
Fig 7.15

% AMYLASE AT 70% INSULIN
Mean of 3 consecutive preparations per digestion method

Fig 7.16

% INSULIN IN PELLET
Mean of 3 consecutive preparations per digestion method
Fig 7.17 Digest x50 produced by AM4. Overdigestion of the Pancreas has Produced Multiple Islet Fragments and only Small Numbers of Intact Islets.

Fig 7.18 Digest x50 Produced by AM2 Containing Small Islets Only.
Fig 7.20  Purified Porcine Islets x100 after AM3 Digestion and 400mOsmol/kg BSA Density Gradient Centrifugation

Fig 7.21  Purified Porcine Islets x200. Architectural Integrity Preserved.
CHAPTER 8

The Large-Scale Isolation of Porcine islets of Langerhans and their Transplantation into Diabetic Recipients

8.1 Introduction
8.2 Methods and Materials
8.3 Results
8.4 Discussion
8.5 Figures and Tables
8.1 Introduction

In order for clinical xenotransplantation to succeed highly purified islet grafts are required (Gray 1990), to prevent the complications of portal vein injection i.e.; portal hypertension and disseminated intravascular coagulation (Cameron 1981, Traverso 1981) The aim of this study was to develop a method for the mass isolation of viable porcine islets for xenotransplantation.

Large-Scale Isolation

Collagenase digestion is the standard method of releasing islets from the pancreas and it has been demonstrated that intraductal delivery of this enzyme can produce large volumes of digest containing islets and dispersed exocrine cells (Gray, 1984). To prepare islets for potential transplantation a second stage is required, to purify the islets and eliminate the contaminating exocrine tissue. It has been shown in the previous two chapters that optimized automated digestion produces the greatest numbers of cleaved islets in the digest, capable of being purified.

A variety of purification techniques have been employed for islet isolation, including hand-picking, serial sieving and density gradient isolation using Ficoll (Sutherland 1976; Gray 1984, Scharp 1987). Although pure islets can be obtained by hand-picking, this method is unsuitable for the large numbers required for human transplantation. Islet yield can be improved by the technique of serial sieving, but this technique is open to infection and there may be significant loss of the tissue on the mesh (Gray, 1984).

One of the major problems of purifying islets from crude pancreatic digest of a large animal is the volume which must be processed, in order to obtain adequate numbers of purified viable islets. For efficient density gradient isolation, multiple gradient tubes have been required (Scharp, 1987). Although rodent islet isolation can be performed on a test tube basis, the number of gradient tubes required to process the digest from an entire porcine pancreas would be impractical.

In Chapter Five of this study a hyperosmolar discontinuous gradient of bovine serum albumin (BSA) was shown to improve the yield and purity of porcine islets compared with isosmolar BSA. In order to adapt this method to the purification of sufficient islets for xenotransplantation, the difficulties involved in processing a large volume of digest produced from the whole porcine pancreas have to be overcome. A small volume discontinuous density gradient, as described in chapter six for porcine islet
isolation, was adapted for the large-scale purification of porcine islets to determine the possibility of isolating large numbers of porcine islets, sufficient for possible xenotransplantation.

A large volume density gradient can be constructed in a commercial cell separator. The COBE 2991 Cell Separator (Lakewood, Colorado, USA) was developed as a centrifuge to wash units of red blood cells, and to remove the preservative agent from cryopreserved blood (Jones, 1988). More recently, it has been used to purify bone marrow in preparation for transplantation (Gilmore, 1982). The machine consists of a centrifuge bowl, with a flexible membrane in the bottom, into which fits a sealed (sterile) plastic processing bag. The bag has an arrangement of tubing and a special rotating valve which allows fluid to be pumped into it during centrifugation. Beneath the flexible membrane is a hydraulic system which, using an operating system, can displace liquid from the centre of the processing bag through the valve during centrifugation. The arrangement allows rapid production of large volume density gradients. The COBE 2991 has been used successfully in the large-scale isolation of canine and human islets (Lake et al 1989, Alejandro et al 1990). Large-scale isolation of porcine islets, not previously described, was thus attempted with the COBE 2991.

Transplantation into Diabetic Recipients

In order to determine whether the isolated islets were viable, an in vivo, method of assessment was used. Throughout this study it was found impossible to culture islets in tissue culture media overnight without significant tissue disintegration, thus making in vitro insulin-release studies impossible. It was necessary therefore, to transplant the porcine islets immediately into a diabetic animal, in an attempt to show reversal of diabetes, and its return after graft removal. This procedure is the best test of islet function and the ultimate aim of successful islet transplantation. The animal used in these studies was the severe combined immunodeficient (Scid) mouse.

In order to investigate whether the Scid mouse could be usefully used in islet transplantation research, experiments were designed to investigate whether it could be rendered diabetic and accept islet xenografts. Thereafter experiments were designed to determine whether the diabetic Scid mouse could be rendered normoglycaemic by transplantation with porcine islets after large-scale isolation.
8.2 Materials & Methods

Preparation of Pancreatic Digest
Porcine pancreata were obtained as described in Chapter Four. Five pancreata were digested using the automated technique, optimized as described in the last chapter.

Large-Scale Purification
After each digestion, the final digest was spun down to a pellet. 10ml of this washed final digest pellet was thoroughly resuspended in 200ml of 400mosmol/kg BSA of high density, 1.095g/ cm³, in a sterile 500ml glass bottle.

A large volume discontinuous BSA density gradient was formed on the COBE 2991 by first running the digest / BSA mixture under gravity into a processing bag, which had been placed in the centrifuge bowl of the COBE 2991.

Centrifugation was then commenced at 1 000rpm and a second layer of BSA, of isolation density, 100ml 400mosmol/ kg BSA, density 1.075g/ cm³, was loaded onto the gradient, by use of a peristaltic pump (Model 503S, Watson-Marlow, Falmouth, UK).

This was followed by a ‘capping’ layer of BSA, 50ml of 400mosmol / kg BSA of density 1.061g/ cm³. This was added immediately after the 1.075g/ cm³ BSA.
The gradient was completed by the addition of 150ml of 400mOsmol/kg MEM. The complete gradient may be represented as:

- MEM 150ml
- 1.061g/cm³ 400 mOsm/kg BSA 50ml
- 1.075g/cm³ 400 mOsm/kg BSA 100ml
- 1.095g/cm³ 400 mOsm/kg BSA 200ml +10ml Digest

After removal of any air, centrifugation speed was increased to 2000 rpm (800g) for five minutes. The upper 100ml of supernatant MEM was then pumped off to waste and the islet containing interfaces at the MEM / 1.061g/cm³ BSA interface and the 1.061 / 1.075g/cm³ interfaces were collected. Collection was made directly into labelled sterile 250ml conical flasks.

The flasks containing the islets were placed on ice and the islets were washed with MEM / 10% NBCS in 50ml centrifuge tubes.

After two washings, the final islet preparation was resuspended in 50ml MEM / 10% NBCS. Five x 200-μl samples of the final islet preparation were stained with dithizone.
and the islets measured and counted as previously described. Final purity was estimated visually.

The Severe Combined Immunodeficient (Scid) Mouse

The murine Scid mutation was produced at the Fox Chase Cancer Centre in 1980 (Bosma et al 1983). The mutation arose in the C.B-17 inbred strain, a congenic partner of BALB/c. Mice derived from a single breeding pair were shown to be deficient in all the major immunoglobulin (Ig) classes, as well as lacking functional T cells. Myeloid and erythroid lineages were unaffected by the mutation. Selective breeding of Ig negative mice led to the establishment of a colony of mutant mice, and further studies showed that the disorder had an autosomal recessive inheritance. Although homozygous mice lacked mature functional lymphocytes and were highly susceptible to recurring infections, they were otherwise normal.

The Scid defects have subsequently been shown to result from a defective VDJ-recombinase system (Schuler 1986), an enzyme system responsible for the assembly in recombinant fashion of variable (V) diverse (D) and junctional (J) chains to form the variable antigen-binding region of both Ig and T cell receptor proteins. If the VDJ-recombinase system is incompetent, then affected lymphocytes are non functional due to a lack of antigen specific receptors. It has been noted (Carroll 1988) that 10-20% of young adult Scid mice produce detectable serum Ig. These mice have been designated 'leaky' and leakiness increases with age. Scid mice leaky for B cells may also be leaky for T cells, Ig -ve mice may rarely be leaky for T-cells.

Experimental Animals

Scid mice were obtained from the National Institute of Medical Research (Mill Hill, London, UK) and housed in filter-top cages.
Prophylactic co-trimoxazole was not used. Mice were screened between the ages of 6 and 8 weeks for the presence of detectable serum mouse Ig. Mice with detectable mouse Ig (‘leaky’ Scids') were excluded from the study. The incidence of “leakiness” in the colony was 15%.

Diabetes was induced in Scid mice by streptozotocin (STZ, Sigma, Poole, UK) injection intraperitoneally, 100mg/kg on three consecutive days. The induction of diabetes in the SCID mouse by this method had been established by previous work on human immune system reconstitution in this department. The intraperitoneal route of administration was used in order to minimise handling of the animals. The mortality of animals receiving this dosage was approximately 20% but lesser doses did not produce permanent diabetes. Diabetes was confirmed only in animals with a blood glucose >20mmol/l for at least seven days.

**Xenograft Acceptance Testing**

The ability of the diabetic Scid mouse to accept xenografts was tested by the transplantation to the renal subcapsular space of rat islets.

Rat islets were prepared using a standard method of rodent islet isolation (Lake et al 1989) from inbred WAG / Ola rats. 500 islets were transplanted to the renal subcapsular space of six halothane-anaesthetised diabetic SCID mice in a clot of the recipients blood.

Blood glucose was measured 24, & 48 hours after transplant, then twice weekly for three weeks. At three weeks, those mice in whom diabetes had been reversed (blood glucose < 10mmol/l) underwent nephrectomy, under halothane anaesthesia of the graft-bearing kidney. Blood glucose was measured at 24 and 72 hrs post-nephrectomy. The graft-bearing kidney was examined histologically and immunohistologically for insulin and / or evidence of rejection.

**Porcine Islet Viability Testing**

Pure porcine islets were prepared by optimized automated digestion and large-scale isolation as described in this and the previous chapter.

The final islet ‘pellet’ was resuspended in 50ml of MEM / 10% NBCS and poured into a 150mm diameter petri dish, from which islets were handpicked using a drawn glass pipette. Even with preparations of > 90% purity it was possible to increase the purity of the final preparation by this technique.
From the final islet suspension of each preparation, two x 1 000 islet aliquots were hand-picked, spun down at 100g for two minutes and transported on ice to the animal house, to be transplanted within 30 minutes of final isolation.

Under halothane anaesthesia, each aliquot was transplanted beneath the renal capsule of a diabetic Scid mouse. After achieving deep anaesthesia, a drop of blood was obtained from a tail vein and thoroughly mixed with the islet aliquot. A left flank incision was made, the left kidney was exposed and delivered, the capsule incised longitudinally and the islets suspended in the clot placed beneath the capsule. The capsule was replaced over the graft and the wound closed.

Monitoring of graft function was by daily blood glucose measurement (BM Stix, Boehringer, FRG, read by automated glucose sensor) at 8 - 8.30 am at 24, 48, and 72 hours and thereafter twice weekly for 3 weeks. Successful transplantation was defined as the production of a blood glucose level less than 10mmol/l by 1 week post-transplant and its maintenance until nephrectomy.

At 3 weeks the graft-bearing kidney was removed under halothane anaesthesia and examined histologically for presence of functioning islet tissue and / or rejection. Blood glucose was again measured at 48 and 96 hours post-nephrectomy. All mice were killed at completion of these studies.

8.3 Results

All values are median (range).

Digest Production

Five porcine pancreata, mass 63g (54-67g) underwent optimized automated collagenase digestion as described in Chapter Seven. A digest volume of 23ml (19-37ml) was produced, with a cleavage index of 97% (94-98%).

The digest contained 5 877 islets /g pancreas (4896-7184), with a mean diameter of 112µm (108-118µm) and a mean volume of 15.5 x 10^-4 mm³ (11.9-18.2mm³).

The resulting total amount of islet tissue in the digest was 10.2mm³ per gram pancreas (6.23-10.8mm³), giving a total number of 105µm islet equivalents of 16 830 per gram (10280-17820). Full details of this data are given in Table 8.1
Large-Scale Purification

After COBE 2991 large-scale density gradient isolation, using a 400mOsmol/kg BSA gradient of 1.09 to 1.095 g/cm³, the islet-containing interface for each preparation was quantified using the usual techniques.

Pig islets of 88% purity (80-95%) were obtained. A total number of 1649 islets per gram pancreas (1255-2189) were obtained, representing a mean yield of 29.5%.

The mean islet volume of purified islets was 16.1 x10^-4mm³, (9.89-21.9) giving a post-purification islet tissue/g yield of 2.65mm³ (1.79-4.03), representing 30.0% of the total islet tissue present in the amount of digest placed onto the gradient.

Full details of this data are given in Table 9.2 and expressed graphically in Figs 8.1-8.5.

Rat Xenografts

Four Scid mice with confirmed streptozotocin-induced diabetes received 500 freshly isolated rat islets beneath the left renal capsule under halothane anaesthesia.

The results of serial blood glucose measurements are given in Table 8.3. The means of the values were used to construct the graph in Fig 8.6, showing the acceptance by the Scid mice of the rat islet xenografts, by their ability to reverse diabetes until removal at three weeks post-transplant.

Pig Xenografts

Ten Scid mice with confirmed diabetes received 1000 fresh pig islets each, two mice being transplanted with the islets from each of the COBE preparations described in this chapter.

Results of the serial blood glucose measurements are given in Table 8.4. Only the results of the transplants of eight mice are given, as two out of the ten did not have their diabetes reversed by the transplants.

The means of the values in Table 8.4 were used to construct the graph in Fig 8.7, showing reversal of diabetes by the pig islet xenografts.
8.4 Discussion

Attempts to treat patients with insulin-dependent diabetes by transplantation of human islets have only been successful with the transplantation of an adequate islet mass, approx 10,000 islet equivalents/kg body weight (Warnock et al 1991), consisting of relatively pure (>70%) islets (Scharp et al 1989). If advances in xenotransplantation research continue, the transplantation of porcine islets for human diabetes is also likely to be dependent on these same conditions. The studies in the previous chapters of this thesis have outlined the development of a method for effective isolation of porcine islets; by optimized automated digestion and density gradient centrifugation using 400mOsmol/kg bovine serum albumin.

The aim of this study was to determine the possibility of isolating porcine islets on a large-scale density gradient, in sufficient numbers likely to reverse diabetes in experimental animals, and then to determine their viability by attempting to reverse diabetes in such animals. The optimized automated digestion was again successful in producing large, probably intact islets in the digest, producing a mean value of 10.2 mm³ islet tissue per gram pancreas. After purification the final mean yield was 2.65 mm³ islet tissue per gram pancreas, with mean yield after five preparations being approximately 30% of the original islet tissue placed on the gradient, both in terms of islet tissue and islet equivs per gram pancreas. Yield can always be increased at the expense of purity, but with purities at this level of yield averaging 88%, Fig 8.8, it is doubtful if the 'trade' would be worthwhile. An 88% purity is acceptable for transplantation beneath the kidney capsule of a rat (Gray et al 1986), and purities approaching 50% or less are associated with markedly decreased islet function. In fact purities averaging 80% may well be acceptable for human xenotransplantation, if the purities of successful human islet transplants are a guide (Scharp et al 1990). The longest surviving human islet graft, producing insulin independence in a type 1 diabetic patient, is 70% pure (Warnock et al 1991).

In the previous studies comprising this thesis, viability has been difficult to determine as the relatively fragmented islets isolated have been impossible to keep in culture. Their break-up into single cells within hours of culture has prevented effective perfusion studies. The nude rat colony in Leicester has been very difficult to keep healthy and to breed with, so the development of the Scid mouse has been very welcome, enabling formal in vivo viability testing with regard to the reversal of diabetes. This is after all the most important criterion of islet function in the field of transplantation. Studies into the reconstitution of the human immune system in the Scid
mouse conducted at this laboratory (London et al 1991) determined the dosage of streptozotocin for development of diabetes (see above). Experiments also showed that the Scid mouse would accept long-term rodent and human xenografts with reversal of induced diabetes. With 500 human islets being able to reverse diabetes in the diabetic SCID mouse, it was determined to use 1000 porcine islets in similar experiments to allow for any isolation damage to the more fragile porcine islets and the lower mean volume of porcine islets. Standard hand-picking techniques were used but as the experiment was not designed to test pig islets versus rat islets no precautions against inherent bias were taken.

Diabetes was successfully reversed in eight of the ten mice. Although two grafts failed to reverse diabetes, they were from different islet preparations; the other paired transplant from the same preparation successfully reversed the diabetes in each case. This suggests that the problem was technical rather than the result of impaired islet viability. The results of the remaining eight transplants showed that prompt reversal of diabetes occurred, reflected in both the serum glucose levels and weight gain. Normoglycaemia was maintained for twenty one days and removal of the grafts resulted in return of hyperglycaemia to diabetic levels. Histological examination of grafts from successfully transplanted animals showed a band of endocrine tissue beneath the renal capsule, with no evidence of rejection. It is interesting to note that the blood glucose level after graft removal seldom returned to the highest levels attained before transplantation. As blood glucose levels were only monitored for 48 hours after graft removal it is possible that the previous levels of hyperglycaemia might have been reached with more time. The transplantation of 1000 porcine islets beneath the renal capsule of a mouse is a fairly delicate manoeuvre, it is also very possible that islets were split into the peritoneal cavity and therefore not removed at nephrectomy.

For effective treatment of diabetes by islet transplantation blood glucose levels must not only be maintained within normal limits, but the grafted tissue must be able to respond physiologically to a glucose load. The performance of glucose tolerance testing was attempted but thwarted by the technical problems of repeated venous access in the mouse. The blood glucose monitoring of the grafts required thirteen separate tail venepunctures per mouse, and after repeated puncture / tail cutting the intravenous administration of glucose was not found to be a practical possibility.

In conclusion a method for the large scale isolation of intact, (Fig 8.9), porcine islets using bovine serum albumin density gradients is described. The islets isolated are
viable, and capable of reversing diabetes when transplanted as xenografts into a suitably receptive diabetic host.
Table 8.1 DIGEST ISLET QUANTIFICATION

<table>
<thead>
<tr>
<th>Preparation No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mass (g)</strong></td>
<td>62</td>
<td>56</td>
<td>67</td>
<td>63</td>
<td>63</td>
</tr>
<tr>
<td><strong>Digest Volume (ml)</strong></td>
<td>22</td>
<td>26</td>
<td>37</td>
<td>19</td>
<td>23</td>
</tr>
<tr>
<td><strong>Cleavage (%)</strong></td>
<td>98</td>
<td>94</td>
<td>97</td>
<td>97</td>
<td>92</td>
</tr>
<tr>
<td><strong>Islets per gram pancreas</strong></td>
<td>5877</td>
<td>7134</td>
<td>5963</td>
<td>4896</td>
<td>5237</td>
</tr>
<tr>
<td><strong>Mean Diameter (μm)</strong></td>
<td>112</td>
<td>118</td>
<td>109</td>
<td>108</td>
<td>118</td>
</tr>
<tr>
<td><strong>Mean Islet Volume x10^-4 mm³</strong></td>
<td>17.4</td>
<td>14.2</td>
<td>18.2</td>
<td>18.5</td>
<td>11.9</td>
</tr>
<tr>
<td><strong>Islet Tissue per gram pancreas (mm³)</strong></td>
<td>10.23</td>
<td>10.2</td>
<td>10.8</td>
<td>7.59</td>
<td>6.23</td>
</tr>
<tr>
<td><strong>105μm Islet Equivalents per gram pancreas</strong></td>
<td>16881</td>
<td>16832</td>
<td>17822</td>
<td>12525</td>
<td>10281</td>
</tr>
</tbody>
</table>
Table 8.2 POST-PURIFICATION ISLET QUANTIFICATION

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<th>5</th>
</tr>
</thead>
<tbody>
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<td><strong>Yield Isolated</strong></td>
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<td>37.3</td>
<td>35.2</td>
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<tr>
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<td><strong>Isolated Islets per gram pancreas</strong></td>
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<td>1649</td>
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<tr>
<td><strong>Mean Diameter Isolated Islets (μm)</strong></td>
<td>122</td>
<td>107</td>
<td>126</td>
<td>107</td>
<td>94</td>
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<tr>
<td><strong>Mean Islet Volume x10^-4 mm³</strong></td>
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<td>9.9</td>
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<td><strong>Islet Tissue per gram (mm³)</strong></td>
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<td>1.79</td>
<td>4.03</td>
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<td><strong>105μm Islet Equivalents per gram</strong></td>
<td>4290</td>
<td>2954</td>
<td>6650</td>
<td>4405</td>
<td>3565</td>
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<tr>
<td><strong>Purity (%)</strong></td>
<td>90</td>
<td>80</td>
<td>95</td>
<td>85</td>
<td>90</td>
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</table>
Fig 8.1

MEAN ISLET VOLUMES

Volume (x10^-4 mm³)

<table>
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<tr>
<th>PREPARATION No.</th>
<th>DIGEST ISLET VOLUME</th>
<th>PURIFIED ISLET VOLUME</th>
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<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
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<tr>
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</table>

0.000 | 0.001 | 0.002 | 0.003

Legend:
- □ DIGEST ISLET VOLUME
- □ PURIFIED ISLET VOLUME
Fig 8.2

**ISLET EQUIVALENTS PER GRAM PANCREAS**

![Bar chart showing islet equivalents per gram across different preparations.](chart1)

- Digest Islet Equivs per gram
- Purified Islet Equivs per gram

Fig 8.3

**% YIELD AS ISLET EQUIVALENTS / GRAM PANCREAS**

*After optimized automated digestion and COBE 2991 purification*

![Bar chart showing % yield of islet equivalents per gram across different preparations.](chart2)
Fig 8.4

**ISLET TISSUE PER GRAM PANCREAS**

<table>
<thead>
<tr>
<th>PREPARATION NO.</th>
<th>Digest Islet Tissue per gram</th>
<th>Purified Islet Tissue per gram</th>
</tr>
</thead>
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<td>1</td>
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<td>2</td>
</tr>
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<td>2</td>
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</tr>
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<td>8</td>
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% YIELD AS ISLET TISSUE PER GRAM PANCREAS

**Fig 8.5**

% YIELD AS ISLET TISSUE PER GRAM PANCREAS

AFTER OPTIMIZED AUTOMATED DIGESTION AND COBE 2991 PURIFICATION

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Table 8.3  RAT ISLET XENOGRAFT DATA

Blood Glucose Values in mmol/l

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<td>14.3</td>
<td>18.3</td>
<td>19.5</td>
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</tr>
</tbody>
</table>

Fig 8.6

RAT ISLET XENOGRAFTS TO Scid MOUSE RECIPIENTS

Mean (+SEM) of 4 Recipient Mice

Blood Glucose (mmol/l)

- Transplant 500 islets
- Nephrectomy
Table 3.4

**Porcine Islet Xenografts to Diabetic Scid Mice**

*Blood Glucose Values in mmol/l*

<table>
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<tr>
<th>Mouse No:</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
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<td>28.3</td>
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</table>
Fig 8.7

PIG ISLET XENOGRAFTS TO DIABETIC SCID MICE

Transplant 1000 Pig Islets

Mean (+SEM) values of 8 Transplants

BLOOD GLUCOSE (mmol/l)

Nephrectomy

DAY
Fig 8.8 Porcine Islets x100 at a Purity of Approximately 90%, after Optimized Automated Digestion and Large-Scale Isolation

Fig 8.9 Porcine Islet x200 after Large-Scale Isolation. Diameter 150μm.
Fig 8.10  The COBE 2991 Cell Separator
Conclusions and Future Work

This thesis has addressed some of the problems inherent in the isolation of porcine islets of Langerhans; attempting to develop a method which can produce tissue capable of reversing diabetes in an animal model.

Collagenase digestion is the only useful way of disaggregating the pancreas into its component tissues, and no better method than density gradient centrifugation has yet been found for purification of the islets from this dispersed tissue.

The manual biopsy-based method of collagenase digestion described in Chapter Four has not previously been described for the porcine pancreas and it was hoped that it might be able to produce the pure, whole islets necessary for islet transplantation. The results presented showed that it does not produce a digest from which islets can be purified using isosmolar BSA. Before discarding this method the effect of the osmolality of the BSA was tested. It had been noticed in the laboratory during work on human islet isolation, that the efficiency of islet purification varied between batches of BSA. Investigations into the cause of this variation revealed marked differences in osmolality between BSA solutions from different sources. These variations ranged from 290 to 550 mOsmol/kg water. Thus it seemed likely that the tonicity of the purification medium was having an effect. The effect was tested in the experiments of Chapter Five. It was seen from the results that increasing tonicity of BSA was associated with a differential increase in the density of porcine exocrine tissue over that of islets, resulting in improved islet purity which was maximal at 400mOsmol/kg H$_2$O.

Following from the work of Ricordi et al it was decided to develop an automated method of digestion. Encouraging results had been reported with this technique but no direct comparison using identical tissues, had ever been made between the automated and another method. The results presented in Chapter Six showed that a digest of greatly increased ‘quality’ was produced with the automated method, a greater quantity of islet tissue being produced in the digest. It was disappointing that this digest was still not capable of better purification on the now osmotically optimized BSA density gradient. It was noted that the mean diameter of ‘purified’ islets was still not as great as the mean histological diameter, meaning that significant fragmentation was occurring with the automated method. This technique was initially quite violent, with prolonged violent shaking of the digestion chamber being thought necessary. The poor results of the purification experiments in Chapter Six thus led to the testing of modifications of automated digestion made in Chapter Seven. It was found that less violent automated
digestion dramatically improved both the amount of islet tissue in the digest (by avoiding islet fragmentation) and the purity of isolated islets after BSA density gradient centrifugation. It is interesting to note that these findings have recently been supported by work from the Canadian group (Lakey 1992) who showed that in the canine pancreas the use of a gentler automated technique resulted in an increased islet tissue yield.

The production of enough tissue to reverse diabetes in an animal model remained untested, leading to the experiments of Chapter Eight. It was found possible to produce enough viable porcine islets to reverse the disease in a diabetic immunodeficient mouse, using the methods developed during these studies.

Improvements in porcine islet isolation will probably be based, at least in the short-term, on improvements on the two mechanisms of collagenase digestion and density gradient centrifugation.

The possession of an efficient collagenase is possibly the most important factor in the islet isolation process. The search for the 'perfect enzyme' occupies every laboratory working in the field. The variability of activity amongst batches remains one of the biggest problems in islet isolation, often preventing useful work for months until a good enzyme is found. It is hoped that some consistency may be produced in the future, perhaps by means of molecular engineering.

Isopycnic density gradient centrifugation is the most widely used technique for the purification of islets from human and large mammalian pancreata. The efficiency of islet isolation by this method is highly variable and considerable scope for improvement exists. The best medium remains to be found but the best results are currently being produced with Euro-Ficoll or BSA. Euro-Ficoll may be better than BSA and is the subject of many on-going studies. It is hoped that the effect of the newer organ-preservation solutions will be tested on porcine tissue as they are developed. Changes in the physical and chemical environment of cells affect their cell volume, and hence their density and behaviour on a density gradient. Temperature, osmolality and the ionic composition of the medium are thus important, and studies are needed on determination of the optimal conditions of purification. Porcine islet purification has recently been noticed to dependent on the temperature of the gradient medium (Chadwick 1993)
The porcine tissue used for islet isolation in the future may not necessarily be that of the standard abattoir pig used in these studies. Although good results have been obtained with this pig, other centres are reporting good results with pigs of different ages and breeds. Much work remains to be done on the optimization of the porcine tissue which is to subject to islet isolation. The period of warm ischaemia inherent in pancreatic retrieval at present needs to be minimized, and may lead to loss of the temperature effect noted above, as the islets are then less susceptible to hypothermia-induced swelling. The effect of in-situ perfusion of organ preservation solutions before pancreatic retrieval has not yet been adequately studied.

It is hoped that the disease of Type 1 diabetes mellitus may be prevented in the future by immunological means. Until that time, the refinement of the techniques of the transplantation of insulin-secreting tissue should continue. There is little sign at present of any increase in the available number of human organs, for either pancreatic or islet transplantation. There is thus an enormous shortfall between supply and demand for this tissue, which can only be made up by the use of porcine pancreata. The major barrier to the use of porcine islet tissue in the treatment of diabetes in the human is of course the xenograft barrier but current opinion indicates that the future of clinical xenotransplantation lies in the use of highly unrelated donor species (Najarian 1992). Another problem lies in the field of porcine islet preservation and culture where significant advances have yet to be made. If immunological and tissue culture advances continue, porcine islet xenografting will become a realistic therapeutic option in the treatment of diabetes.
APPENDIX 1

Insulin Radioimmunoassay

Reagents

**RIA Buffer**
Radio-immunoassay buffer. 0.5% BSA (Sigma A7030, Lot 127F-0384) in PBS.

**Serum**
Guinea pig anti-bovine serum (ICN Biomedicals Ltd, High Wycombe, Bucks).

**125I Insulin**
3-Iodotyrosyl A14 Insulin, human, recombinant freeze dried solid. (IM166 activity 1.85 MBq, Amersham International Ltd, Amersham, UK). 20ml aliquots in 5-6ml of RIA buffer gave 20-30 counts per tube.

**Sac cell**
Donkey anti-guinea pig coated cellulose suspension (IDS, Washington, UK), diluted 1:2 in PBS.

**Wash solution**
0.1% Triton X-100 (Sigma T6878) in distilled water.

**Human Insulin Standards**
Human serum (Novo Biolabs Ltd, Cambridge, UK). 40ml of human serum in 960ml of RIA buffer is 8ng/ml, serial dilutions were carried out to give 4,2,1,0.5,0.25,0ng/ml.

**Equipment**

LP3 tubes and stoppers (SS.483, Sarsseadt, Numbrecht, Germany).
Centrifuge - CR 422, Jouan, Tring, UK.
Gamma Counter _ 1282 Comugama CS,LKB, South Croydon,UK).
Vortex-Genie (Scientific Industries, New York, USA).
Methods

1. Samples were diluted appropriately with RIA buffer and each tube was assayed in duplicates.

LP3 tubes were used, and for each assay the same series of controls, standards and samples were used.

<table>
<thead>
<tr>
<th>Tubes 1&amp;2</th>
<th>Total counts (50ml of $^{125}$I insulin)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tubes 3&amp;4</td>
<td>Blanks (50ml serum)</td>
</tr>
<tr>
<td>Tubes 5-19</td>
<td>Human Insulin Standards + 50ml serum</td>
</tr>
<tr>
<td>Tubes 20-181</td>
<td>50ml of sample + 50ml serum</td>
</tr>
</tbody>
</table>

2. Each tube was vortexed and stored at 22°C for 30 minutes.

3. 50ml of $^{125}$I insulin was added to each tube and left after vortexing for 90 minutes at 22°C.

4. 50ml of Sac cell was added to tubes 3-181, vortexed again, and left for a further 30 minutes at 22°C.

5. 1ml of wash solution was added to each tube, and the tubes centrifuged at 500g for 6 minutes (no brake). The supernatant was tipped off and the tubes capped.

6. The samples were then counted in a gamma counter which calculated a standard curve and the insulin content of the samples in ng/ml, using the Multicalc Data management Package (Pharmacia, Milton Keynes, UK). The principle involved subtracting the mean blank count rate from the mean count rate of each set of duplicate samples. By comparison with a log plot of the % inhibition of the total counts in the insulin standards, inhibition of the total count was used to determine the insulin concentration in the samples.
APPENDIX 2

Amylase Assay

This procedure uses the hydrolysis of a water-insoluble cross-linked starch polymer carrying a blue dye by α-amylase to form water-soluble blue fragments.

Reagents
Human α-amylase control.
Starch polymer in tablet form (Phadebas Amylase Test - Pharmacia Diagnostics, AB Uppsala, Sweden).
0.5M NaOH (S/4845/60, Fisons).

Equipment
10 ml conical test tubes (144AS, Sterilin).
Cuvettes (67.742, Sarstedt, Numbricht, Germany).
Spectrophotometer - SP 1800, Pye Unicam, Cambridge, UK.

Procedure
1. 4 ml of distilled water was pipetted into 10ml conical tubes.
2. 200μl samples, at appropriate dilutions, were added to the tubes, including distilled water & MEM as blanks and human α-amylase at 1/5 dilution as control.
3. The tubes were pre-incubated at 37°C for at least five minutes in a waterbath.
4. Starch polymer was added in tablet form using forceps. Each tube was then immediately vortexed for 10 seconds and replaced in the waterbath.
5. Tubes were incubated for exactly 15 minutes at 37°C.
6. 1 ml of 0.5N NaOH was added to each tube and vortexed to stop the reaction.
7. Tubes were centrifuged at 1500 G for 5 minutes.
8. Absorbance of the supernatant was measured at 620nm against distilled water, using plastic cuvettes, with 1 cm light path.
9. The absorbance of the blank was subtracted from that of the samples and the values were then read from the date coded standard curve supplied with each Phadebas kit.
Appendix 3

Composition of Solutions Used

Collagenase

<table>
<thead>
<tr>
<th>Volume</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>100ml</td>
<td>MEM</td>
</tr>
<tr>
<td>100mg</td>
<td>Collagenase (Batch 03092C, Serva) 1-mg/ml.</td>
</tr>
<tr>
<td>1.5ml</td>
<td>1 M Ca CI2</td>
</tr>
<tr>
<td>10ml</td>
<td>DNA-ase (Sigma, DN 25)</td>
</tr>
</tbody>
</table>

After sonication for 20 minutes this was filter sterilized.

Dithizone

<table>
<thead>
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<th>Volume</th>
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</thead>
<tbody>
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<td>190mg</td>
<td>Dithizone (Sigma D5130, f wt.256.3) dissolved in:</td>
</tr>
<tr>
<td>30ml</td>
<td>Dimethyl sulfoxide (DMSO).</td>
</tr>
</tbody>
</table>

After mixing overnight, filter and add 20ml of the filtrate to 180ml MEM containing 2% NCS mixing continuously during the addition.

MEM

<table>
<thead>
<tr>
<th>Volume</th>
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<tr>
<td>400ml</td>
<td>10 x MEM</td>
</tr>
<tr>
<td>80ml</td>
<td>Penicillin &amp; Streptomycin 100 U/ml</td>
</tr>
<tr>
<td>40ml</td>
<td>Fungizone</td>
</tr>
<tr>
<td>40ml</td>
<td>HEPES 10mmol</td>
</tr>
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</table>

Added to 3360ml sterile water.

For MEM used in the circuit of the automated digestion technique, 2% NCS was added.
Density gradient centrifugation is the standard method for isolating human islets from the collagenase digested pancreas. However, islet purity varies considerably between pancreata.

The osmolality of the media used for the density gradient purification of human islets ranges from 290 mOsm (e.g. Seromed Ficoll) to 550 mOsm (e.g. dextran, Ficoll DL). The purpose of this study was to determine whether osmolality had an effect on the purity of human islets isolated by density gradient centrifugation.

Ten consecutive human pancreata were disrupted by collagenase digestion and aliquots of the digest were placed on continuous bovine serum albumin (BSA) density gradients of osmolality 290 mOsm or 500 mOsm. The osmolality of these gradients did not vary throughout the gradient. The density of the 290 mOsm gradient ranged from 1.051 - 1.076 g/ml and the 500 mOsm gradient from 1.070 - 1.102 g/ml. The continuous gradients were centrifuged at 800g for 20 min and eleven consecutive 1 ml aliquots removed from each gradient and extracted for amylase and insulin content.

The peak density of insulin/islets in the 295 mOsm BSA gradients was 1.061 g/ml for all ten pancreata, whilst the peak density of amylase/exocrine tissue ranged from 1.061 - >1.076g/ml. The peak density of islets in 500 mOsm BSA was not the same for each pancreas and ranged from 1.082 - 1.090 g/ml; the peak density of exocrine tissue ranged from 1.092 - >1.102 g/ml.

The median (range) percent of amylase tissue present at the density of BSA needed to obtain 95% of the insulin for the 290 mOsm gradients was 51.0% (4.8-95.0), and for the 500 mOsm gradients 6.4% (1.0-81.0); p<0.05.

Thus the density of human islets does not vary in BSA of physiological osmolality, however the density of exocrine tissue varies markedly and this explains the variable purity of human islet preparations. In hyperosmolar (500 mOsm) BSA the peak density of both islets and exocrine tissue varies, but the purity of the final islet preparation is significantly better than in BSA of physiological osmolality.
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